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Director
Prof. Claudio Pignata

PhD Thesis

Primary Immunodeficiencies: novel insights in pathogenesis and potential therapeutic approaches

Student
Dr. Loredana Palamaro

Tutor
Prof. Claudio Pignata

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BACKGROUND AND AIMS

The immune system detect a wide variety of agents, from viruses to parasitic worms, and distinguish them from the organism's own healthy tissue. Disruption of any part of the orchestrated immune response can result in an inability to control infections and subsequent illness. Apart from physical barriers, the immune response is composed from a diverse network of defenses, including cellular components and soluble mediators. A proper immune response relies on the innate immunity, characterized by a rapid and nonspecific initial response to infections and later on the adaptive immunity, characterized by a specific response to a particular antigen. The innate immune response involves three major cell types: phagocytic cells, such as neutrophils and macrophages, natural killer (NK) cells and antigen presenting cells (APC), which are also involved in the induction of an adaptive immune response. The adaptive immune system includes T and B lymphocytes responsible for cellular and humoral responses, respectively. However, these components of the immune system act in a well orchestrated and integrated unique system in order to maintain a normal resistance to infections. Failure of host defense cause the dysregulation of the immune system, in particular the onset of immunodeficiency, autoimmunity and cancer predisposition.

Primary immunodeficiencies (PIDs) comprise more than 200 different disorders that affect the development and the functions of the immune system. In most cases primary immunodeficiencies are monogenic disorders that follow a simple mendelian inheritance. Primary immunodeficiencies are rare and have an overall prevalence of approximately 1:10.000 live births and are classified according to the component of the immune system that is primarily involved [1]. Defects in

adaptive immune responses include antibody deficiency syndromes and combined immunodeficiencies (CIDs). Defects of innate immunity comprise disorders of phagocytes, Toll-like receptor (TLR)–mediated signaling, and complement. All of these forms are characterized by increased susceptibility to recurrent infections, severe infections, or both, with distinctive susceptibility to various types of pathogens depending on the nature of the immune defect. In addition, some forms of PIDs present with immune dysregulation, and others (immunodeficiency syndromes) have a more complex phenotype in which immunodeficiency is only one of multiple components of the disease phenotype [2].

PIDs are often associated with various autoimmune manifestations [2]. For many years autoimmunity and immune deficiency have been considered two opposite extremes of immune function but it is now clear that they represent intertwined phenomena that reflect inadequate immune function [3,4]. In these cases the autoimmunity does not seem to be related to a defect of tolerance to self-antigens, but rather to a persistent stimulation as a result of the inability to eradicate antigens [5]. This immune dysregulation leads to compensatory and exaggerated chronic inflammatory responses that result in tissue damage and autoimmunity.

Moreover, primary immunodeficiencies are usually characterized by an increased susceptibility to cancer. In the last decade cancer has become the most significant life-threatening complication of PIDs after infections [6,7]. In fact the major life span observed in patients affected with PIDs thanks to the improvements in the prevention and treatment of infections also led to an increased incidence of malignancies. A high cancer susceptibility has been reported for some types of PIDs, such as ataxia-telangiectasia (AT), common variable immunodeficiency (CVID), Wiskott-Aldrich Syndrome (WAS), SCID, selective IgA deficiency, DNA repair deficiencies and Hyper IgM Syndromes. The main type of malignancy in PIDs is non

Hodgkin lymphoma, followed by Hodgkin lymphoma and leukemia [8]. However, the type of malignancy depends on the specific PID, the age and the infective clinical history of the patients. The genetic defect, underlying the immunodeficiency, may play a direct role in cancer pathogenesis either facilitating chronic infection, especially with oncogenic viruses such as Epstein Barr virus (EBV), or through genetic mutations in DNA double-strand breaks repair systems, which lead to accumulation of mutations that promote tumorogenesis; the genetic defect can also play an indirect role in the tumor development which results from the break-down of normal immune surveillance of transformed cells [9,10].

This thesis reports the results obtained during my PhD course in "Human Reproduction, Development and Growth" (XXV Cycle) from March 2010 to March 2013.

My PhD programme has been focused in the study of the following lines of research:

- ✓ Study of the functional role of transcription factor FOXN1 in the T-cell ontogeny and molecular and clinical characterization of a primary T-cell immunodeficiency due to FOXN1gene mutation;
- ✓ Study of an impaired regulation of the immune system;
- ✓ Study of primary immunodeficiencies associated with cancer predisposition and neurodegeneration.

CHAPTER I

"Thymus and T-cell development"

The thymus provides the microenvironment essential for the development of T cells from hematopoietic stem cells (HSCs) [11,12]. An important feature of the thymic microenvironment is its three-dimensional (3D) organization, consisting of an ordered architecture of thymic stromal cells (TSCs), that represent a heterogeneous mixture of cell types including cortical and medullary thymic epithelial cells (TECs), fibroblasts, endothelial cells, dendritic cells (DCs), and macrophages [13,14]. Among these stromal elements, the thymic epithelial cells are the most abundant cell types which forms a delicate 3D cellular network spanning throughout both the thymic cortex and the medulla. The requirement for a 3D-supporting stroma appears to be unique to T cell development, as the *in vitro* development of other hematopoietic lineages, including B cells and NK cells, does not require a 3D structure [15].

The intrathymic development of T cells consists of several phases that require a dynamic relocation of developing lymphocytes within multiple architectural structures of this organ. T-cell progenitors originate in the bone marrow, enter the thymus and, through a series of defined and coordinated developmental stages, differentiate, undergo selection, and mature into functional T cells. The steps in this process are regulated through a complex transcriptional network, specific receptor-ligand pair interactions, and sensitization to trophic factors, which mediate the homing, proliferation, survival, and differentiation of developing T cells.

Following the entry into the thymus through the cortico-medullary junction, lymphoid progenitor cells begin their commitment toward the T-cell lineage. The developmental pathway is traditionally divided into three subsequent steps, as defined by peculiar immunophenotypic patterns: the $CD4^{-}CD8^{-}$ double negative (DN) stage, the $CD4^{+}CD8^{+}$ double positive (DP) stage and the $CD4^{-}CD8^{+}$ or $CD4^{+}CD8^{-}$ single positive (SP) stage. In mice, an immature single positive (ISP) $CD8^{+}CD4^{-}$ cell may be detected between the DN and DP stages (**Figure 1**).

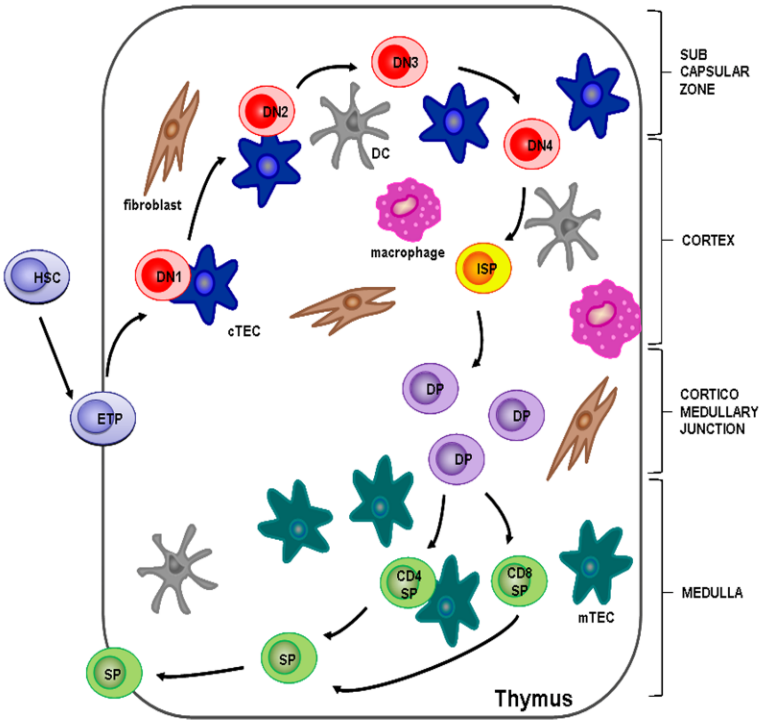

Figure 1. Steps of the T-cell development

The transition to the next stage in development depends on interactions with a complexity of thymic stromal cells and, in particular, the 3D configuration of thymus maximizes this interaction allowing intercellular cross-talk integral to the development of both T cells and TSCs [16-20]. In particular, signals from early $CD4^{-}CD8^{-}$ DN T-cell precursors and/or their immediate progeny provide the signals that promote the formation of the thymic cortex, while, later in ontogeny, the differentiation of thymic epithelial cells to a medullary phenotype and the subsequent formation of a medulla proper are clearly dependent on the presence of $CD4^{+}CD8^{-}$ and $CD4^{-}CD8^{+}$ SP selected thymocytes [18,21,22].

The DN stages transition requires the expression of different arrays of genes, as the induction of recombinase activating gene-1 (RAG-1) and RAG-2, the upregulation of pre-Tα (pTα) and the rearrangement of T-cell receptor (TCR) δ and γ. These cells become competent to undergo β-selection and express the pre-TCR complex on their surface and reach the DN3 stage [23]. After β-selection, the thymocytes, which have properly rearranged TCRβ chains, show a burst of

proliferation and a subsequent upregulation of CD8 and then CD4 and become DP cells. Eventually, DP cells rearrange TCRα gene, leading to TCRα assembly into a TCR complex. In the cortex, the DP thymocytes interact through their TCR with peptide-MHC complexes expressed by stromal cells, as cTECs and dendritic cells [24]. At this site, take place the positive selection, where 'productive' T cells react to foreign antigens, but not to self antigens [25]. Lately, positively selected DP thymocytes are ready to differentiate into SP cells, that is $CD4^{+}CD8^{-}$ or $CD4^{-}CD8^{+}$ and relocate into the medulla. At this site, newly generated SP thymocytes are further selected by the medullary stromal cells, including autoimmune regulator (AIRE)-expressing mTECs. The cells that are reactive to tissue-specific self antigens are deleted, thus avoiding autoimmunity [25]. SP thymocytes egress from the thymus as recent thymic emigrants (RTE), naïve cells. These RTE cells are fully mature T cells that exert proper functional capabilities of cell-mediated immunity [26-28].

§1.1 T-cell development: focus on FOXN1

The thymic microenvironment is composed primarily of an integrated meshwork of cortical and medullary TECs, required for promoting most stages of the thymocyte differentiation [17]. In particular, the epithelial cell-autonomous gene *Foxn1* is required for thymic epithelial patterning and differentiation from the initial epithelial thymic anlage to a functional cTEC and mTEC meshwork during crosstalk with the lymphoid compartment.

FOXN1 gene belongs to the forkhead gene family that comprises a diverse group of 'winged-helix' transcription factors that have been implicated in a variety of biochemical and cellular processes, such as development, metabolism, aging and cancer [29,30].

Foxn1 gene is differentially expressed during the life span. In particular, during the prenatal life the transcription factor plays a critical role in the appropriate development of several epithelia, such as that of liver, lung, intestine, kidney and urinary tract. Postnatally, *Foxn1* is mainly expressed in thymic epithelial cells, some keratinocyte populations and hair follicles, where it acts

through its molecular targets to regulate the balance between growth and differentiation [31-33].

The development and maturation of TEC subsets during the thymus organogenesis occurs through two genetic stages [31,34], first stage being Foxn1-independent and under the control of genes such as Hoxa3 [35] and Tbx1 [36], during which induction and outgrowth of the thymic epithelial anlage from the third pharyngeal pouch take place. In the Foxn1-dependent step, precursor epithelial cells differentiate into mature and functional cTECs and mTECs from the same bipotential TEC progenitor [34,37-39]. Of note, Foxn1 is expressed in all TECs during embryogenesis, but not in all TECs of the adult thymus [40], indicating that the gene is strictly developmentally regulated. Since Foxn1 regulates the undifferentiated TECs, its expression in the adult thymus reveals the presence of TEC progenitors [41], which support TEC homeostasis in the adult thymus.

As far as concerned FOXN1 function in the skin, its role, its target genes and the molecular mechanism of action still remain to be fully clarified. A detailed analysis of the *Foxn1* expression in mouse skin has revealed a specific pattern of expression, suggesting its key role in the regulation of keratinocytes growth and differentiation [32].

However, it is largely unknown whether the role of foxn1 in the thymus and skin is identical. One important difference is that, it has recently been demonstrated that Foxn1 is also involved in the 3D thymic micro-structure morphogenesis and maintenance [42] and, eventually, Foxn1 must be considered as a prime downstream mediator of agents or pathways also capable to induce thymic involution or rebound.

Although FOXN1 has been long studied, most of the studies thus far available are restricted to fetal differentiation process, while its postnatal role in the mature thymus still remains to be fully elucidated.

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Review Article

From Murine to Human Nude/SCID: The Thymus, T-Cell Development and the Missing Link

Rosa Romano, Loredana Palamaro, Anna Fusco, Leucio Iannace, Stefano Maio, Ilaria Vigliano, Giuliana Giardino, and Claudio Pignata

Department of Pediatrics, "Federico II" University, Via Pansini 5, 80131 Naples, Italy

Correspondence should be addressed to Claudio Pignata, pignata@unina.it

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Primary immunodeficiencies (PIDs) are disorders of the immune system, which lead to increased susceptibility to infections. T-cell defects, which may affect T-cell development/function, are approximately 11% of reported PIDs. The pathogenic mechanisms are related to molecular alterations not only of genes selectively expressed in hematopoietic cells but also of the stromal component of the thymus that represents the primary lymphoid organ for T-cell differentiation. With this regard, the prototype of athymic disorders due to abnormal stroma is the Nude/SCID syndrome, first described in mice in 1966. In man, the DiGeorge Syndrome (DGS) has long been considered the human prototype of a severe T-cell differentiation defect. More recently, the human equivalent of the murine Nude/SCID has been described, contributing to unravel important issues of the T-cell ontogeny in humans. Both mice and human diseases are due to alterations of the FOXN1, a developmentally regulated transcription factor selectively expressed in skin and thymic epithelia.

1. Introduction

Primary immunodeficiencies (PIDs) are severe disorders of the immune system in which patients cannot produce a proper protective immune response, leading to an increased susceptibility to infections. Nowadays, more than 200 well-characterized genetic immune deficiencies have been identified thanks to the advances in molecular genetics and immunology. PIDs are classified according to the component of the immune system that is primarily involved including T, B, natural killer (NK) lymphocytes, phagocytic cells, and complement proteins [1].

Primary T-cell defects are rare disorders, accounting for approximately 11% of reported PIDs [2]. These diseases may be considered true experiments of the nature in that the recognition of the molecular mechanisms underlying their pathogenesis led to clarify the phases of the T-cell differentiation process and the physiological mechanisms of the T-cell responses. Studies in this field led to unravel the checkpoints, which play a pivotal role in these processes, which mostly rely on a proper intercellular interaction between thymocytes and the thymic microenvironment.

2. T-Cell Development and Thymus

The thymus is the primary lymphoid organ that supports T-cell differentiation and repertoire selection [3, 4]. The intrathymic development of T cells consists of several phases that require a dynamic relocation of developing lymphocytes within multiple architectural structures of this organ. As shown in Figure 1, these steps are (1) the entry of lymphoid progenitor cells into the thymus, (2) the generation of $CD4^+$ $CD8^+$ double positive (DP) thymocytes in the cortex, (3) the positive selection of DP thymocytes in the cortex, and (4) the interaction of positively selected thymocytes with medullary thymic epithelial cells (mTECs) to complete the thymocyte maturation and, eventually, the export of mature T cells from the thymus [5].

Thymus anlagen arises as bilateral structures from the third pharyngeal pouch in the embryonic foregut [6, 7]. The interaction of the epithelial component with the lymphoid progenitor takes place as early as embryonic day 11.5 in mice and at the eighth week of gestation in humans [8, 9].

At an early stage, these precursors have both lymphoid and myeloid potential [10, 11] and are characterized by

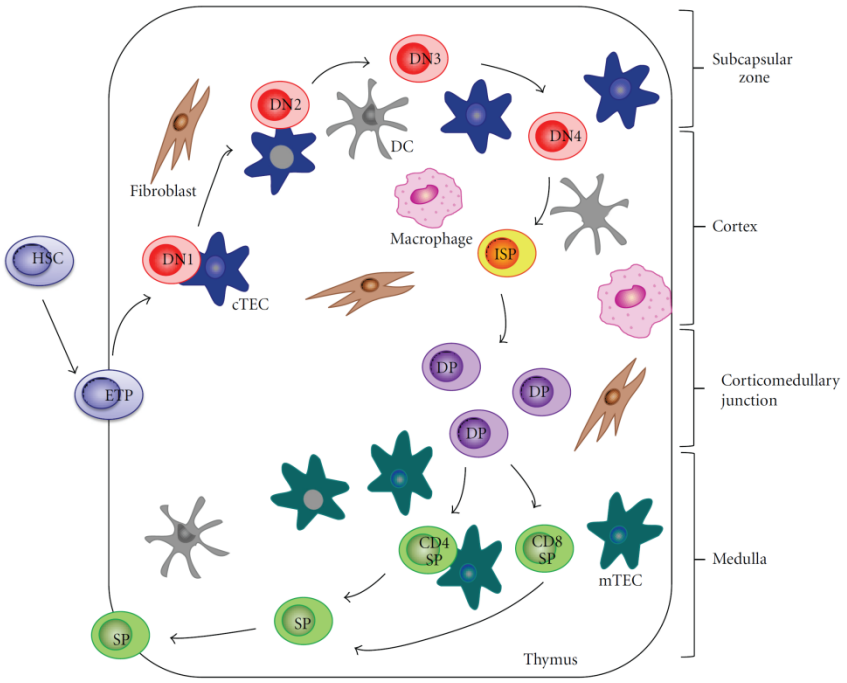

Figure 1: Steps of T-cell development. The lymphoid progenitor cell goes into the thymus through the cortico-medullary junction. DN thymocytes ($CD4^-CD8^-$) migrate across the subcapsular region and then the outer cortex. Interaction between DN cells and cTECs generates DP thymocytes ($CD3^+CD4^+CD8^+$). Positively selected thymocytes interact with mTECs to complete the maturation process. In the medulla, self-reactive thymocytes are deleted, SP ($CD3^+CD4^+$ or $CD3^+CD8^+$) thymocytes are generated, and, eventually, the export of mature T cells from the thymus takes place.

the expression of the CC-chemokine receptor 9 (CCR9), that, along with the CCR7, plays a central role in this precocious stage of thymus colonization. At this stage of differentiation, lymphoid cells also express the stem- and progenitor-cell markers KIT (also known as CD117), the stem-cell antigen-1 (SCA-1), and the growth-factor-receptor tyrosine kinase type 3 (FLT3) [12–14].

Following the entry into the thymus through the corticomedullary junction, lymphoid progenitor cells begin their commitment toward the T-cell lineage. The developmental pathway is traditionally divided into three subsequent steps, as defined by peculiar immunophenotypic patterns: the $CD4^-CD8^-$ double negative (DN) stage, the $CD4^+CD8^+$ double positive (DP) stage, and the $CD4^-CD8^+$ or $CD4^+CD8^-$ single positive (SP) stage. In mice, an immature single positive (ISP) $CD8^+CD4^-$ cell may be detected between the DN and DP stages. This population can be easily distinguished from the mature SP cell by the high levels of expression of T-cell receptor (TCR) β and CD3 and the low level of CD24 (heat stable antigen, HSA). DN cells in mice can be further subdivided based on the expression of CD44 and CD25 in the following populations: $CD44^+CD25^-$(DN1), $CD44^+CD25^+$ (DN2), $CD44^-CD25^+$(DN3), and $CD44^-CD25^-$(DN4) [15].

From the early T-cell lineage progenitor (ETP) stage to the double-negative 3 (DN3) stage, T-cell differentiation is independent from the TCR and is dependent on the migration through the distinct thymic structures [16]. These phases are regulated by the expression levels of specific transcription factors and by a fine tuned interplay between them (Figure 1).

At the beginning, ETPs and DN2 cells exhibit a high proliferative capability. Differently, at the DN3 stage, when a fully rearranged TCR occurs, the proliferation stops. In the initial thymocyte development till the DN3 stage, Notch-mediated signals play a pivotal role [17, 18] also supported by signals delivered through the interleukin-7 receptor (IL-7R) [19, 20].

The immature thymocytes journey through the thymus has also the additional effect of promoting the differentiation of thymic stromal precursors into mature thymic epithelial cells, thus playing an important role in the formation of

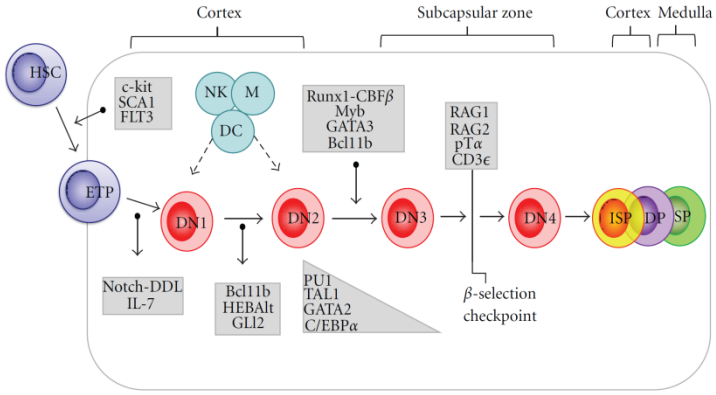

Figure 2: Differential gene expression profile, which modulates the discrete stages of the T-cell development. The lymphoid progenitors, entering into thymus and expressing the markers of HSCs, are primed to Notch and IL-7 signaling until DN1 stage. During the transition DN1/DN2, immature thymocytes lose multilineage potential through the downregulation of genes involved in the differentiation towards other cellular lineages, as PU.1, TAL1, GATA-2, and C/EBPα. At the DN2 stage, Myb, GATA-3, HEBalt, GLI-2, and Bcl-11b are upregulated. At the DN3 stage, the genes required for a proper TCR assembly as Rag-1, Rag-2, and pTα are expressed, thus leading to the β-selection. Following β-selection check-point, DN4 cells are fully committed to the TCRαβ$^+$ T-cell lineage.

the thymic microenvironment [21–24]. In particular, thymocytes during the DN1-DN3 stages participate to the differentiation process of TEC precursor cells into cortical TECs (cTECs).

The DN1 cell thymocytes keep the potential to differentiate into B, T, myeloid, NK, and dendritic cells (DCs) [25–27]. The transition to DN2 is characterized by the upregulation of a number of genes involved in the process, including genes needed for rearrangement and/or expression of the pre-TCR signaling complex components (Figure 2) [28]. At this stage, the thymocytes lose the multilineage potential due to silencing of genes involved in the differentiation towards other cellular lineages. Nevertheless, this potential is not completely lost, since cells with the DN2 phenotype can still differentiate into NK cells, DCs, or macrophages under certain circumstances [29, 30].

DN2 stage T cells are fully responsive to IL-7 and SCF due to the high expression of IL-7Rα and c-kit. The DN2 stage is characterized by the upregulation of CD25 molecule (interleukin-2 receptor α, IL-2Rα) and CD90 (Thy-1) [28]. Moreover, the genes which favor the myeloid, NK, and dendritic fate, so-called T-cell antagonists, as PU.1, stem-cell leukemia (SCL also known as TAL1), GATA binding protein-2 (GATA-2), and CCAAT-enhancer binding protein α (C/EBPα) are silenced before that β or γδ selection takes place (Figure 2) [31]. During this phase only a few transcription factors, including the zinc-finger transcription factor, the tumor suppressor factor B-cell lymphoma/leukemia 11b (BCL-11b) [32], basic helix-loop-helix (bHLH) transcription factors alternative (HEBalt) [33], and, more transiently, glioma-associated oncogene 2 (GLI-2), a transcription factor involved in the sonic hedgehog signaling [34], are expressed (Figure 2).

The following DN2 to DN3 stage transition requires the expression of different arrays of genes, as Runt-related transcription factor 1-Core binding factor β (Runx1-CBFβ) complexes, the transcription factor Myb, GATA-3, and Bcl-11b, which allow full TCRβ gene rearrangement in thymocytes, that become competent to undergo β-selection [35–37]. Several important events occur during the DN2/3 transition, as the induction of recombinase activating gene-1 (Rag-1) and Rag-2, the upregulation of pre-Tα (pTα), and the rearrangement of TCRδ and γ. CD3ε and IL-7Rα (CD127) are also upregulated at this phase [38] along with the turn-on of the *lck* tyrosine kinase implicated in the pre-TCR and TCR signaling [39]. At this point, T-cell precursors lose their capability to follow a non-T-cell fate choice [28].

The cells overcoming β-selection express the pre-TCR complex on their surface and reach the DN3 stage [40]. Thereafter, the E-proteins E2A and HEB play a crucial role in several processes and are required for the progression of the T-cell development. In fact, these proteins are involved in the TCR gene rearrangement [41], in conferring the competence to undergo β-selection, and in the arrest of thymocyte proliferation at the DN3 stage [42].

At the DN3 stage, pre-TCR signaling results in the downregulation of CD25, pTα, Rag-1, and Rag-2, which leads to the appearance of DN4 cells. These cells are fully committed to the αβ T-cell lineage [43, 44]. After β-selection, the thymocytes, which have properly rearranged TCRβ chains, show a burst of proliferation and a subsequent upregulation of CD8 and then CD4. At this point, the cells become double positive (DP). Eventually, DP cells rearrange TCRα gene, leading to TCRα assembly into a TCR complex.

The newly generated DP thymocytes are localized in the cortex and express low levels of the TCRαβ complex. This

DP population consists of T cells with an unselected repertoire [45, 46]. Following that, positive and negative selections take place. In the cortex, the DP thymocytes interact through their TCR with peptide-MHC complexes expressed by stromal cells, as cTECs and dendritic cells [47]. When TCR interacts with low-avidity with the peptide-MHC ligands, DP thymocytes receive survival signals. This process, referred to as positive selection, allows "productive" T cells to potentially react to foreign antigens, but not to self-antigens [5]. Lately, positively selected DP thymocytes are ready to differentiate into SP cells, that is, $CD4^+CD8^-$ or $CD4^-CD8^+$ and relocate into the medulla. At this site, newly generated SP thymocytes are further selected by the medullary stromal cells, including autoimmune regulator- (AIRE-) expressing mTECs. The cells which are reactive to tissue-specific self antigens are deleted, thus avoiding autoimmunity [5]. SP thymocytes egress from the thymus as recent thymic emigrants (RTEs), naïve cells expressing the CD62 ligand (CD62L), also known as lymphocyte- (L-) selectin, CD69, and the CD45RA isoform. These RTE cells are fully mature T cells that exert proper functional capabilities of cell-mediated immunity [48–50].

3. Pathogenetic Mechanisms of T-Cell Defects

Most of the pathogenic mechanisms underlying primary T-cell disorders are related to molecular alterations of genes selectively expressed in hematopoietic cells. However, since the differentiation process requires a crosstalk among thymocytes and thymic microenvironment, a severe T-cell defect may also be due to alteration of the stromal component of the thymus.

T-cell disorders include a wide spectrum of disorders that affect T-cell development and/or function. The severity of the T-cell defect varies a lot ranging from the syndrome of severe combined immunodeficiency (SCID), characterized by a complete absence of T-cell functions to combined immunodeficiency disorders, in which there are a low number of T cells whose function is not adequate [51].

SCIDs comprise a heterogeneous group of monogenic disorders characterized by a virtual lack of functional peripheral T cells. To date, more than 20 different genetic defects involved in the pathogenesis of SCID in humans have been identified [52, 53]. Typically, patients with SCID show a severe defect in T-cell differentiation and a direct or indirect impairment of B-cell development and function. On the basis of the involvement of different cell lines in the pathogenesis of the disease and of the subsequent different clinical phenotypes, SCIDs have been till now classified according to the presence or absence of T, B, and NK cells (Table 1). Impaired survival of lymphocyte precursors is observed in reticular dysgenesis (RD) and in adenosine deaminase (ADA) deficiency. In RD the mutations of the adenylate kinase 2 gene (AK2) result in increased apoptosis of myeloid and lymphoid precursors. As a consequence, patients with RD show marked lymphopenia and neutropenia [54, 55]. ADA deficiency is characterized by the accumulation of high intracellular levels of toxic phosphorylated metabolites of adenosine and deoxyadenosine that cause apoptosis of lymphoid precursors in the bone marrow and thymus [56, 57].

Table 1: SCIDs classification. SCIDs have been so far classified according to the presence or absence of T, B, and NK cells, as a consequence of different molecular defects.

Lymphocyte phenotype	Gene defect	Form of SCID
$T^-B^-NK^-$	Adenylate kinase	Reticular dysgenesis
	Adenosine deaminase	ADA deficiency
$T^-B^+NK^-$	IL-2Rγ	SCID-X1
	Jak3	SCID-AR
$T^-B^+NK^+$	IL-7Rα	IL-7Rα deficiency
$T^-B^-NK^+$	Rag-1 or Rag-2 artemis	Omenn syndrome Artemis deficiency

The majority of SCIDs in human subjects derive from alterations of the cytokine-mediated signaling apparatus. SCID-X1 represents the most common form of SCID and is caused by mutations of the IL-2 receptor γ gene (IL-2Rγ), which encodes for the common γ-chain (γ-c) shared by cytokine receptors, including those for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. Patients usually have few or no T and NK cells but a normal or elevated number of B cells which fail to produce immunoglobulins normally [58]. γ-c also plays effects on cell cycle control and participates to the growth of tumoral cells, as well [59, 60]. Defects of JAK3, an intracellular tyrosine kinase physically and functionally coupled to γ-c, result in a syndrome whose immunologic phenotype is undistinguishable from that of SCID-X1 [61]. Mutations in the gene encoding for the α-chain of the IL-7R abrogate T lymphocyte development but leave B and NK cell development intact [62]. Mutations in critical genes needed for the expression of pre-T-cell receptor, as Rag-1 and Rag-2, result in a functional inability to form antigen receptors through genetic recombination, compromising the production of functional T cells. These proteins recognize recombination signal sequences and introduce a DNA double-stranded break, permitting V, D, and J gene rearrangements [63, 64]. Lymphocyte phenotype differs from those of patients with SCID caused by γ-c, Janus kinase-3 (Jak-3), IL-7Rα, or ADA deficiencies in that they lack both B and T lymphocytes since pre-TCR and pre-B-cell receptor (BCR) share similar molecular mechanisms requiring Rag-1 and 2 expression [65]. Defects of pre-TCR and pre-BCR expression might also reflect mutations in genes that encode proteins involved in nonhomologous end-joining (NHEJ) and DNA repair and, in particular, Artemis, DNA protein-kinase catalytic subunit (DNA-PKcs), Cernunnos/XLF, and DNA ligase IV [65–69]. In all these diseases, the generation of both T and B lymphocytes is severely compromised. However, it should be noted that a functional T-cell defect may also be due to infections [70, 71] or during the reconstitution phase following stem cell transplantation [72].

It is noteworthy that all the genes whose alterations lead to the above mentioned forms of SCID selectively impair

the lymphocyte functionality and the ability of these cells to proceed in the developmental pathway. In some cases, as in the case of TrkA mutation [73], the gene has pleiotropic effects resulting in complex multisystemic disorders associated to immunodeficiency.

4. The Murine Model of Athymia: nu/nu Mice

The first example of SCID not primarily related to a hematopoietic cell abnormality but rather to an intrinsic thymic epithelial cell defect is the Nude/SCID phenotype, whose identification contributed to unravel important issues of T-cell ontogeny.

The "nude" phenotype, identified for the first time in mice, results from inactivating mutations in a single gene, originally named winged-helix-nude (whn) and recently known as forkhead box n1 (foxn1) [74]. This murine model was described by Flanagan in 1966, when spontaneously appeared in the Virus Laboratory of Ruchill Hospital in Glasgow (UK) [75–77]. Mice homozygous for the mutation "nude" are hairless, have retarded growth, decreased fertility, and die by 5 months of life for infections. The hairlessness is due to the coiling of the incomplete hair shafts in the dermis caused by the absence of free sulfhydryl groups in the midfollicle region [78]. The "nude" *foxn1* gene does not affect the growth of hair follicles, but the epidermal differentiation process, regulating the balance between proliferation and differentiation of keratinocytes in the hair follicle [79, 80]. The "nude" mice are affected by severe infertility and show small ovaries with low egg counts in the females and no motile sperm in the males [78]. This condition may be the result of changes in hormonal status, as demonstrated by altered serum levels of estradiol, progesterone, and thyroxine [81]. The thymus is absent at birth [82] and there are very few lymphocytes in the thymus dependent areas of the spleen and lymph nodes [83].

Since the abnormal, or even absent, thymus is the hallmark of the "nude" phenotype, these animals develop a profound T-cell deficiency and a severely impaired immune response of either cell-mediated and, indirectly, humoral immunity. In "nude" mice, when the thymus is present in the first days of life, it reveals no normal structure, consisting of a thymic rudiment composed of vesicles or canaliculi delimited by epithelial-like cells, with no trace of lymphoid cells. By the day 14, the "nude" thymus is much smaller compared to the normal [84].

Nu/nu mice show lymphopenia and also low immunoglobulin levels. In the absence of normal T cells originated from the thymus, the development of the antibody forming cells is delayed, although "nude" mice do not lack precursors of antibody forming cells. This indicated that antibody forming cells may mature in the absence of the thymus, albeit at a slower rate [85]. In "nude" mice lymph nodes, the outer cortex with primary nodules and the medullary cords are normal. In the spleen sections from the nu/nu mice, the proportion of red to white pulp is greater than normal and, in some cases, an unusually high number of megakaryocytes are seen in the red pulp. In some spleens, Malpighian follicles, although present, are fewer and smaller than in controls and a depletion of lymphocytes is constant in the close proximity of the central arteriole in the thymus-dependent area. The depletion in the splenic thymus-dependent areas is not as prominent as in the lymph nodes [83]. In man, the prototype of an athymic disorder has long been considered the DiGeorge's Syndrome (DGS), even though main features of athymic murine model and human disease, including immunological signs, are not completely overlapping.

5. The Athymic DiGeorge Syndrome

The DGS, along with velocardiofacial syndrome and conotruncal anomaly face syndrome, is frequently associated to a common heterozygous intrachromosomal deletion in 22q11.2. However, a DGS-like phenotype can have alternative etiologies, including maternal diabetes, fetal alcohol syndrome, and teratogenesis, even though the molecular mechanisms underlying these forms are still unknown [86]. DGS has an estimated incidence of 1 in 4000 live births [87, 88] and, thus, it is the most common microdeletion syndrome in humans and the second most common chromosomal disorder after Down's syndrome. The deletion is due to a meiotic nonallelic homologous recombination between flanking 250 kilobases (kb), mapping in 22q11.2 chromosomal region and consisting in low-copy repeats/segmental duplications in the termed LCR22 [89, 90]. Although most cases of DGS occur as *de novo* deletions, approximately 5% of cases are inherited as an autosomal dominant trait [91–93]. In the 90% of patients, a hemizygous 3 Mb deletion, containing about 30 genes [89, 90, 94, 95], is found, whereas approximately 8% of patients carry a smaller deletion of 1.5 Mb, encompassing 24 genes [96], even though no difference in the clinical presentation is appreciable in the smaller deletion [86].

The main features of this syndrome are mild facial dysmorphism, submucous cleft palate, velopharyngeal insufficiency, speech delay, recurrent infections, variable immunodeficiency secondary to thymic aplasia or hypoplasia, and cardiac anomalies [97, 98]. Most of the patients have learning disabilities and behavioral disorders, including schizophrenia in some cases [99–102]. Children with the DGS, according to the aplasia or hypoplasia of the thymus, are classified as complete or partial DGS. The "complete" form represents a small percentage of patients, accounting to the 0.5% of all patients. These patients show a severe combined immunodeficiency phenotype with near absent T lymphocytes. The majority of patients have a "partial" phenotype and an immune defect usually manifesting as mild to moderate T lymphocytopenia. The T-cell proliferation is usually normal or in very few cases low normal. These patients have been reported to have a moderate increase of the number of infections than predicted on the basis of the immunological impairment, suggesting that anatomical defects, gastroesophageal reflux, allergies, cardiac disease, and poor nutrition may also contribute to recurrent infections [103]. It should be underlined that never "partial" DGS patients have severe infections as reported in SCID and, moreover, T-cell proliferation is usually normal. A moderate CD4 lymphocytopenia with low to normal CD8 T lymphocytes is usually found. An age-related decrease of T lymphocytes is also seen in DGS patients. TCR

repertoire analysis in 22q11.2 deletion patients has shown significant oligoclonal peaks and Vβ family dropouts when compared to controls. In a study of nine patients with a negative infectious history, a decreased diversity in $CD4^+$ and $CD8^+$ TCR repertoire, using both flow cytometric and third complementarity determining region (CDR3 spectratyping) fragment analysis, has been documented [104]. In another study, the spectratyping showed alterations in the repertoire, which, however, improved over the time [105].

Immune deficiency in these patients seems to be associated to an increased incidence of autoimmune diseases [106–108], in particular cytopenias [109, 110], arthritis [111], and endocrinopathies [112].

The chromosomal region usually deleted contains several genes, which may be candidate of the DGS phenotype. TBX1, which belongs to the family of T-box transcription factors, which share a common DNA binding domain is called "T-box" [113]. A specific role for Tbx1 in DGS and thymus development came out from the peculiar expression pattern in both the third pharyngeal pouch endoderm and the adjacent mesenchyme and not in the neural crest cells [114]. Furthermore, the homozygous loss of *Tbx1* causes thymic hypoplasia, as well [96, 115–117]. Of note, mice heterozygous for a null allele of *Tbx1* demonstrate only a mild phenotype without thymus anomalies [118]. Thus, evidence would suggest, at least in mice, that gene dosage of *Tbx1* is crucial in the pathogenesis of DGS. However, in the same region there are other genes potentially implicated in the pathogenesis of DGS, such as *Crkl*, which encodes an adaptor protein implicated in growth factor and adhesion molecule signaling. Homozygous *Crkl* gene deletion results in multiple defects in neural crest derivatives including aortic arch arteries, thymus, and craniofacial structures [96] and in prenatal death. However, the deletion at the heterozygous state does not cause any clinical sign, thus indicating that a combination of gene alterations is needed for the full expressivity of the phenotype [119].

6. The Human Nude/SCID Phenotype

The human equivalent of the "nude" murine phenotype was first described in two sisters in 1996, after more than 30 years from the initial mouse description and, subsequently, associated to *FOXN1* gene alterations.

The human Nude/SCID is an autosomal recessive disorder [120], whose hallmark is the T-cell immunodeficiency due to the complete absence of the thymus. This immunodeficiency presents in a quite similar fashion to the classical SCID phenotype, thus being more severe than DGS. Along with the severe infections, other features of the syndrome are ectodermal abnormalities, as alopecia and nail dystrophy [121]. Of note, the nail dystrophy can be observed also in subjects carrying the genetic alteration in heterozygosity. The most frequent nail alteration is the koilonychia (spoon nail), characterized by a concave surface and raised edges of the nail plate, associated with significant thinning of the plate itself; a canaliform dystrophy associated to a transverse groove of the nail plate (Beau line) may also be found (Figure 3). However, the most specific phenotypic alteration is leukonychia, characterized by a typical arciform pattern resembled to a half-moon and involving the proximal part of the nail plate. These alterations of digits and nails have also been reported in a few strains of "nude" mice. FOXN1 is known to be selectively expressed in the nail matrix where the nail plate originates, thus confirming that this transcription factor is involved in the maturation process of nails and suggesting nail dystrophy as an indicative sign of heterozygosity for this molecular alteration [121].

Interestingly, additional studies have also reported on anomalies of brain structures, suggesting a potential role of this transcription factor in brain embryogenesis, as also suggested by its expression in epithelial cells of the developing choroids plexus, a structure filling the lateral, third, and fourth ventricles. However, the severe neural tube defects, including anencephaly and spina bifida, have been only inconstantly reported, thus probably indicating that the genetic alteration represents a cofactor and is not sufficient *per se* to alter brain embryogenesis. The anomalies of brain structure have been considered potentially responsible for the high rate of mortality *in utero* observed in the geographic area with the high frequency of *FOXN1* alteration [122].

Prenatal alteration of the *FOXN1* gene in humans prevents the development of the T-cell compartment as early as at 16 weeks of gestation [123]. By contrast, stem cells, B, and NK lymphocytes are normal. $CD4^+$ cells are more affected than $CD8^+$ cells, even though the latter are also profoundly reduced. No $CD4^+CD45RA^+$ naive cells can be usually found [123]. CD8 cells coexpressing CD3 are very scarce and a few $CD3^+CD8^+CD45RA^+$ naïve cells can be detected [123]. Overall, a substantial reduction of T cells bearing TCR$\alpha\beta$, but not of lymphocytes expressing TCR$\gamma\delta$, is observed [123]. TCR gene rearrangement, although altered, occurs to some extent, suggesting the possibility of an extrathymic and FOXN1-independent site of differentiation. However, it should be emphasized that these few T cells, which escape the blockage, are unable to sustain a productive immune response into the periphery.

Taken together, the data so far available underline the crucial role of FOXN1 in the early prenatal stages of T-cell ontogeny in humans [123].

7. Role of FOXN1 in Immune System

FOXN1 belongs to the forkhead-box gene family that comprises a diverse group of "winged helix" transcription factors implicated in a variety of cellular processes: development, metabolism, cancer, and aging [124]. These transcription factors share the common property of being developmentally regulated and of directing tissue specific transcription and cell fate decisions. While during embryogenesis *FOXN1* is expressed in several mesenchymal and epithelial cells, including those of the liver, lung, intestine, kidney, and urinary tract, later, its expression is confined to skin and thymus epithelia, where FOXN1 is absolutely required for the normal differentiation of hair follicles and TECs.

FOXN1 gene, spanning about 30 kb [125, 126], is an epithelial cell-autonomous gene and is highly conserved in sequence and function in rodents and humans. Interestingly,

(a) (b) (c)

Figure 3: Nail dystrophy patterns in subjects carrying heterozygous mutations in FOXN1 gene: (a) koilonychias, (b) canaliform dystrophy, and (c) leukonychia.

an extensive screening of cDNA clones obtained from skin cells revealed the presence of two different noncoding first exons [126], the exons 1a and 1b, that undergo to alternative splicing to either of two splice acceptor sites of the exon 2, located upstream of the initiation codon. This suggests the presence of two distinct promoters of exons 1a and 1b [125]. The alternative usage of the exon 1a or 1b seems to direct the tissue specificity [126], in that promoter 1a is active in thymus and skin, while promoter 1b is active only in skin.

The molecular mechanisms by which *FOXN1* expression and activity are regulated are only incompletely understood. It is suggested that FOXN1 might, subsequently, upregulate the expression of fibroblast growth factor (FGF) receptors, which in turn modulate the thymic stroma differentiation and thymopoiesis [127]. *In vitro* exposure of thymic epithelial cells to some Wnt proteins is sufficient to upregulate FOXN1 protein expression in both an endocrine and paracrine fashion [128]. Wnts belong to a large family of secreted glycoproteins that have important roles in cell-fate specification [127].

The prenatal thymus development, the maintenance of a proper thymic microenvironment, and the efficient T-cell production require an appropriate crass-talk between thymocytes and thymic stromal cells [12]. Postnatally, the thymic involution results in dramatically reduced T-cell generation in an age-dependent fashion [129].

Indeed, recent evidence has implicated both TEC- and hematopoietic stem cell- (HSC-) intrinsic defects in involution of the organ [130–133]. *Foxn1* is expressed in all TECs during initial thymus organogenesis and is required for the initial phase of their differentiation [75, 134, 135]. *Foxn1* exerts an important role [136] in inducing both cortical and medullary differentiation [137, 138]. Although foxn1 has long been studied, most of the studies thus far available are restricted to fetal differentiation process, while its postnatal role in the mature thymus still remains to be fully elucidated.

However, it is largely unknown whether the role of foxn1 in the thymus and skin is identical. One important difference is that foxn1 is involved in morphogenesis of the three-dimensional thymic microstructure, which is important for the functionality of the thymus [139]. Moreover, the differentiation of the immature epithelial cells into functional cTECs and mTECs is foxn1-dependent. In particular, foxn1 mainly regulates TEC patterning in the fetal stage [140] and TEC homeostasis in the postnatal thymus [141]. TECs are implicated in either thymus organogenesis or in most stages of maturation of thymocytes [142, 143]. The inborn null mutation in *foxn1* [76] causes a differentiation failure in TECs thereby halting thymic development at a rudimentary stage. The thymic lobar architecture is still present but the epithelial cells lack the ability to induce the entrance of hematopoietic precursor cells (HPCs) into the epithelial cluster and thus preclude the generation of thymocytes [144]. These results argue strongly for a failure in thymocytes-epithelial crosstalk, thus, explaining the blockage of thymic lymphopoiesis [75, 136]. The organ is, therefore, an alymphoid two-dimensional (2D) rudiment with a cystic structure [72, 82, 120, 123].

Because of the significant expression levels of FOXN1 in skin elements, keratinocytes have been successfully used to support a full process of human T-cell development in vitro, resulting in the generation of mature T cells from HPCs. This finding would imply a role for skin as a primary lymphoid organ [145].

8. Conclusion and Future Research

Primary T-cell defects are rare disorders, accounting for approximately 11% of reported PIDs. These disorders include a wide spectrum of diseases that affect T-cell development and/or function. The pathogenic mechanisms are mostly related to molecular alterations of genes selectively expressed in hematopoietic cells. However, they can also be due to alterations of the stromal component of the thymus, which is the primary lymphoid organ that supports T-cell differentiation and repertoire selection. In this organ, the dynamic relocation in multiple architectural structures requires the cross-talk between thymocytes and thymic microenvironment. The Nude/SCID syndrome results from inactivating mutations in the gene encoding the *FOXN1* transcriptional factor selectively expressed in skin and thymic epithelia. In mice and humans its alteration leads to thymic agenesia and severe T-cell deficiency. The Nude/SCID immunodeficiency is much more severe than DGS, indicating that the *FOXN1* expression is absolutely required for an efficient production of mature T cells. The studies on the human Nude/SCID

phenotype greatly contributed to unravel important issues of the T-cell ontogeny and, in the near future, may help define potential extrathymic and thymus-independent sites of differentiation in man.

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§1.2 FOXN1 and human diseases

Most of the pathogenic mechanisms underlying primary T-cell disorders are related to molecular alterations of genes selectively expressed in hematopoietic cells. However, since the differentiation process requires a crosstalk among thymocytes and thymic microenvironment, a severe T-cell defect may also be due to alteration of the stromal component of the thymus. The first example of a Severe Combined Immunodeficiencies (SCID) not primarily related to an abnormality intrinsic of the hematopoietic cell, but rather to a peculiar alteration of the thymic epithelial cell [43,44] is the Nude/SCID Syndrome (MIM 601705; Pignata Guarino Syndrome), due to the absence of FOXN1 transcription factor, that results, both in mice and humans, in congenital athymia and hairlessness. The genetic alteration of the transcription factor, inherited as an autosomal recessive disorder, leads to a severe T-cell immunodeficiency, congenital alopecia of scalp, eyebrows and eyelashes. This phenotype, referred as Nude/SCID, was described for the first time in humans in 1996 in two sisters originating from a small community in the south Italy [43]. This phenotype is widely accepted as the human equivalent of the similar murine phenotype, reported for the first time by Flanagan in 1966 [45].

The Nude/SCID immune deficiency is characterized by a severe blockage of the T-cell differentiation and absence of proliferative response to the common mitogens [43]. Naive T cells are lacking in the peripheral blood, as a consequence of the absence of a T-cell differentiation process. there is no curative therapeutic approach for Nude/SCID syndrome. Bone marrow transplantation has been performed in one child with the Nude/SCID phenotype due to FOXN1 deficiency but without the production of the naïve T-cell pool and long lasting immunological reconstitution [46]. This would be expected given the absence of a functional thymus and to the necessary role of the FOXN1 transcription factor for a proper T-cell development. Nude/SCID phenotype is also characterized by nail abnormalities and variable abnormalities of the central nervous system, such as multiple-site neural tube defects or only the absence of the corpus callosum and cavum septi pellucidi and an enlargement of the interhemispheric fissure, have

been reported suggesting that FOXN1 may be implicated as a co-factor in the development of vital systems required for a proper fetus development [47]. This would explain the high mortality rate during the first trimester of pregnancy reported among consanguineous carriers of the mutation, which is not justified by the SCID per se due to the maternal protection until the third month after birth.

These data have been published as Review on *International Reviews of Immunology*.

§1.3 New strategy to develop an *in vitro* thymic organoid

In congenital immunological disorders and, in particular, in athymic disorders, a scaffold mimicking the 3D structure of primary lymphoid organs may be potentially used for the differentiation of hematopoietic cell precursors and, eventually, allows the re-setting of immunological response through functional or molecular manipulation of precursor cells. An important feature of the thymic microenvironment is its 3D organization, consisting of an ordered architecture of TSCs, through which the developing thymocytes migrate and mature [48]. This 3D configuration maximizes the interaction of developing thymocytes with the supporting stromal cells, allowing a proper intercellular cross-talk integral to the development of both T cells and TSCs [16].

A remarkable number of similarities are shared between the epithelial and stromal cells of the thymus and keratinocytes and fibroblasts of the skin. In particular, both thymic and skin epithelial cells selectively express the FOXN1 transcription factor, which plays a critical role in differentiation and survival of these specialized cells [49-51]. By contrast, a major difference between thymus and skin is the architecture of each organ, in that the skin epithelial cells are mostly distributed along a basement membrane differently from epithelial cells of the thymus, which are organized in a 3D configuration.

It has been recently documented that 3D tantalum-coated carbon matrix is able to support the development of functional T cells from hematopoietic precursor cells in the context of a heterogeneous multicellular system [52], even though the capability of the system to ensure a complete process, resulting in mature type T cells, is still under debate [53].

The *ex vivo* reproduction of microenvironments of the native tissue through approaches based on the use of porous and biodegradable matrices has been proven useful to repair or replace tissues damaged at molecular or functional level [54]. This approach has been successful in achieving the regeneration of many tissues, such as skin [55], cornea [56], blood vessels [57] and in bone replacement process [58,59].

Optimal scaffold materials should exert the properties of excellent biocompatibility, suitable microstructure, controllable biodegradability and suitable mechanical properties to sustain and facilitate a proper intercellular connection [60,61]. Scaffold surfaces need to be organized to allow optimal cell-cell contact, growth, maintenance of morphology and viability over time to meet the demands of the specific application [62].

We verified the hypothesis whether cellular elements of the skin, spatially arranged in a 3D polycaprolactone (PCL) architecture, can support in the absence of thymic components the survival of HSCs and their differentiation into T lineage-committed cells.

The 3D organization is unique to thymic epithelial cells, in that in other organs, epithelial cells are "polarized" and placed on a basal lamina, forming sheets of cells lining internal and external surface [42,63]. The peculiar 3D organization of TECs creates a proper microenvironment, which allows thymocytes migration and a tight lympho-stromal interaction [48,64]. Fetal thymic stromal cell monolayer cultures (TSMC) are not able to support T lymphopoiesis, thus indicating that the 3D structure is required [63].

Recently, a thymic organoid has been engineered by seeding tantalum-coated carbon matrix with thymic murine stroma [65]. This composite was able to generate mature functional T cells from bone marrow derived hematopoietic progenitor cells. In our study, for tissue engineering a different material, as the PCL, has been chosen, since it is a biocompatible structure with a high surface area/volume ratio due to its high porosity, thus being an ideal scaffold (**Figure 2)**.

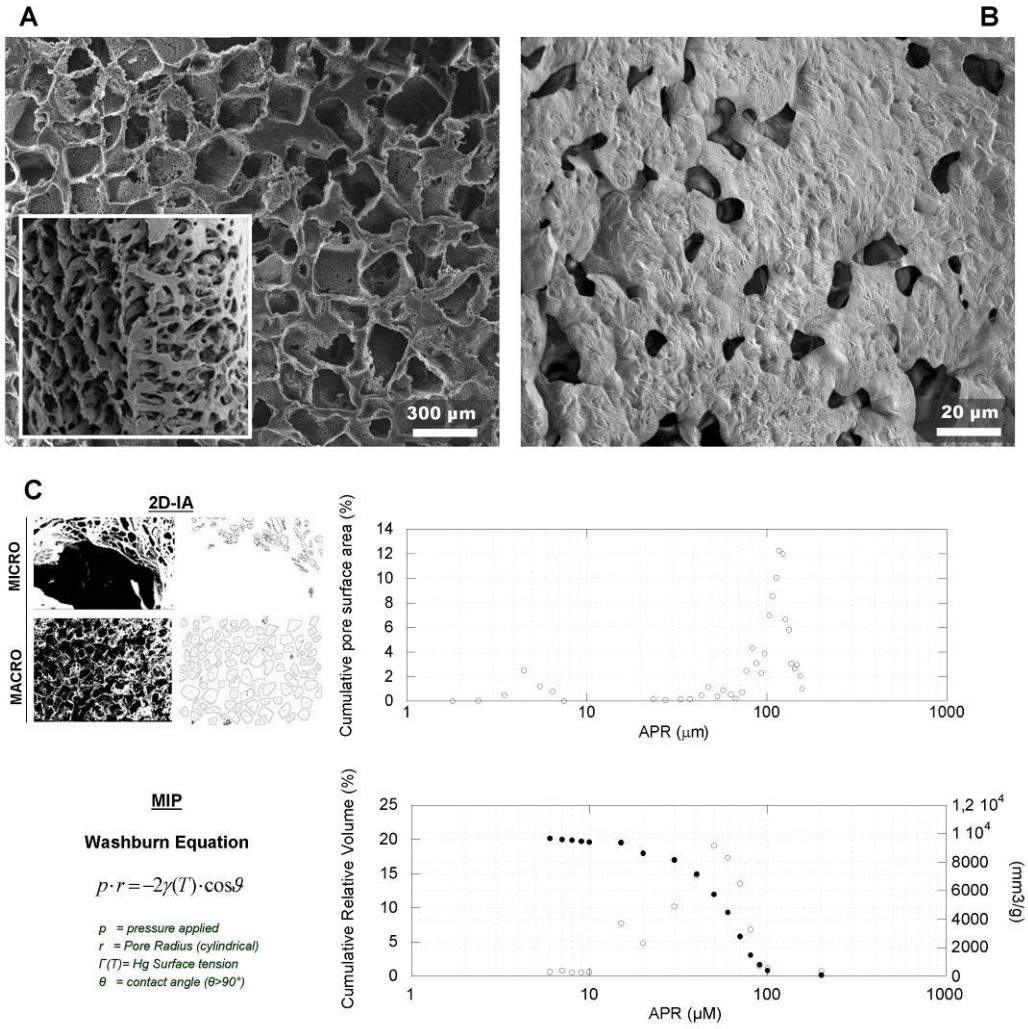

Figure 2 Qualitative and quantitative investigation of PCL scaffold morphology. Scanning electron micrographs of macropores (cross-section in the square) (A) and micropores (B). **(**C) Cumulative distribution of pore surface and volume estimated by 2D-IA and MIP techniques, respectively. APR: average pore radius. Scale bar: 20 and 300 µm.

In addition, the bimodal population of pores is strongly desired to assure an efficient nutrient transport and waste removal within the scaffold. In addition, cell growth and migration are also favoured thanks to a higher surface/volume ratio [66]. Our findings are in keeping with previous observations, which indicate that PCL scaffolds are suitable in guiding cell growth and in facilitating the synthesis

of extracellular matrix, thus leading to the formation of functional tissues and surrogate organs [67].

Since skin derived keratinocytes share several similarities with TECs, our data demonstrate that these cells, in the absence of thymic cellular elements, can replace TECs functionality in supporting the T-cell differentiation process.

In particular, to reproduce *in vitro* the thymic microenvironment, previously expanded fibroblasts and keratinocytes were seeded together onto artificial 3D PCL scaffolds and each cell type was capable to interact with both the material and one to each other (**Figure 3**). A strong interaction of keratinocytes with fibroblasts occurred, resulting in skin cells occupancy of overlapping sites on the scaffold. This characteristic is most important for our system in that it ensures a stable interaction essential to reproduce an in vitro “organoid”.

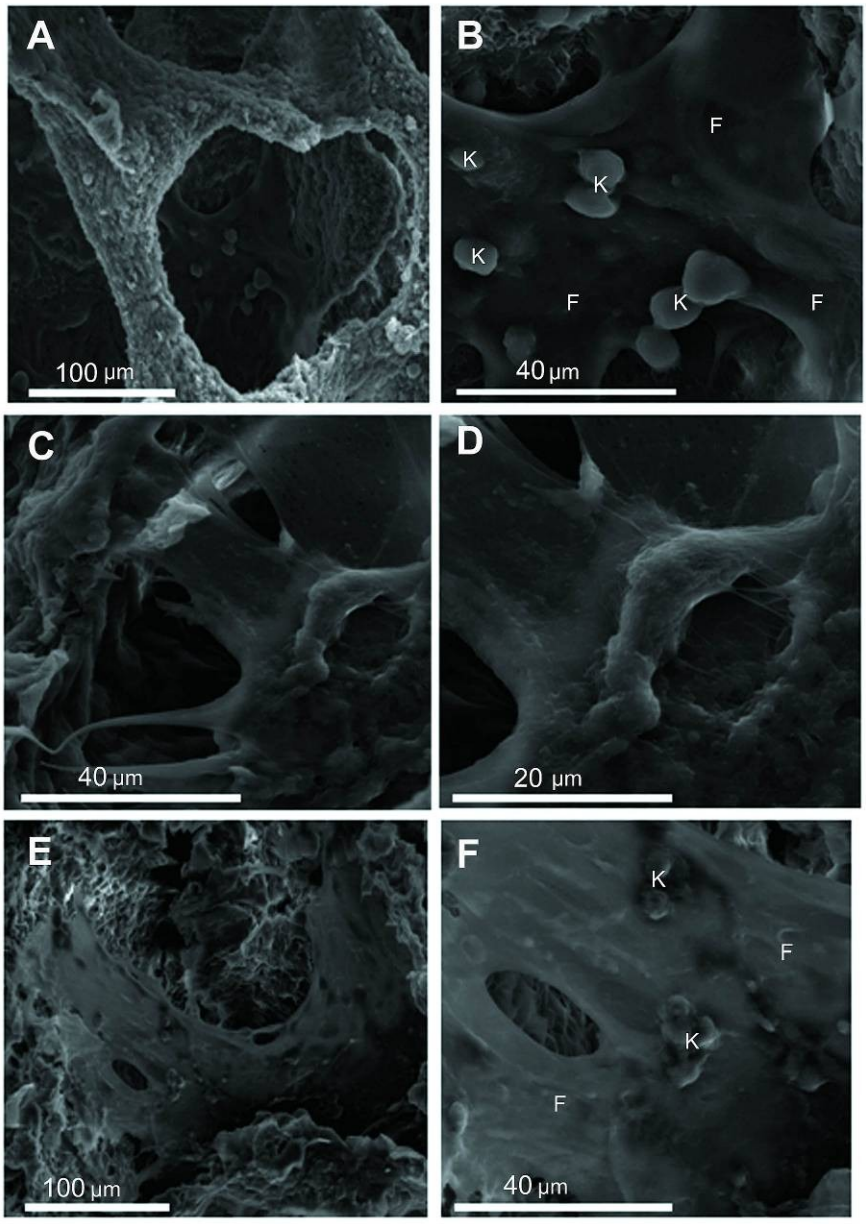

Figure 3. Representative Scanning electron micrographs of keratinocytes and fibroblasts co-coltured on the PCL scaffold. The cells were fixed for 2 h and processed for SEM imaging. Each cell type was capable to interact with the material and one to each other. The panels B, D and F show high magnification SEM images of the panels A, C and E, respectively. Scale bars: 100 µm (A-E), 40 µm (B-C-F), 20 µm (D). F:Fibroblast, K:Keratinocyte. Original magnifications are as follows: panels A and E: X800; panel F: X1600; panel B and C: X 3000; panel D: X6000.

Moreover, to evaluate the importance of the keratinocytes and fibroblasts on CD34+ cells maintenance and differentiation, the cells harvested from the constructs were analyzed by flow cytometry during the 5 w of culture. We demonstrate that the CD34+ cells decreased during the cultures. In the keratinocytes/fibroblasts/PCL scaffold these cells were more represented during the culture and persisted longer than in the control cultures. Moreover, evaluating the behaviour of CD34+ HSCs compared with that of CD45+ in the keratinocytes/fibroblasts/PCL scaffold, we showed that, differently from HSCs, CD45+ cells persisted in the culture at least for 5 w, thus suggesting that in the presence of the multicellular biocomposite, rearranged in a 3D configuration, stem cells undergo to a differentiation process (**Figure 4**).

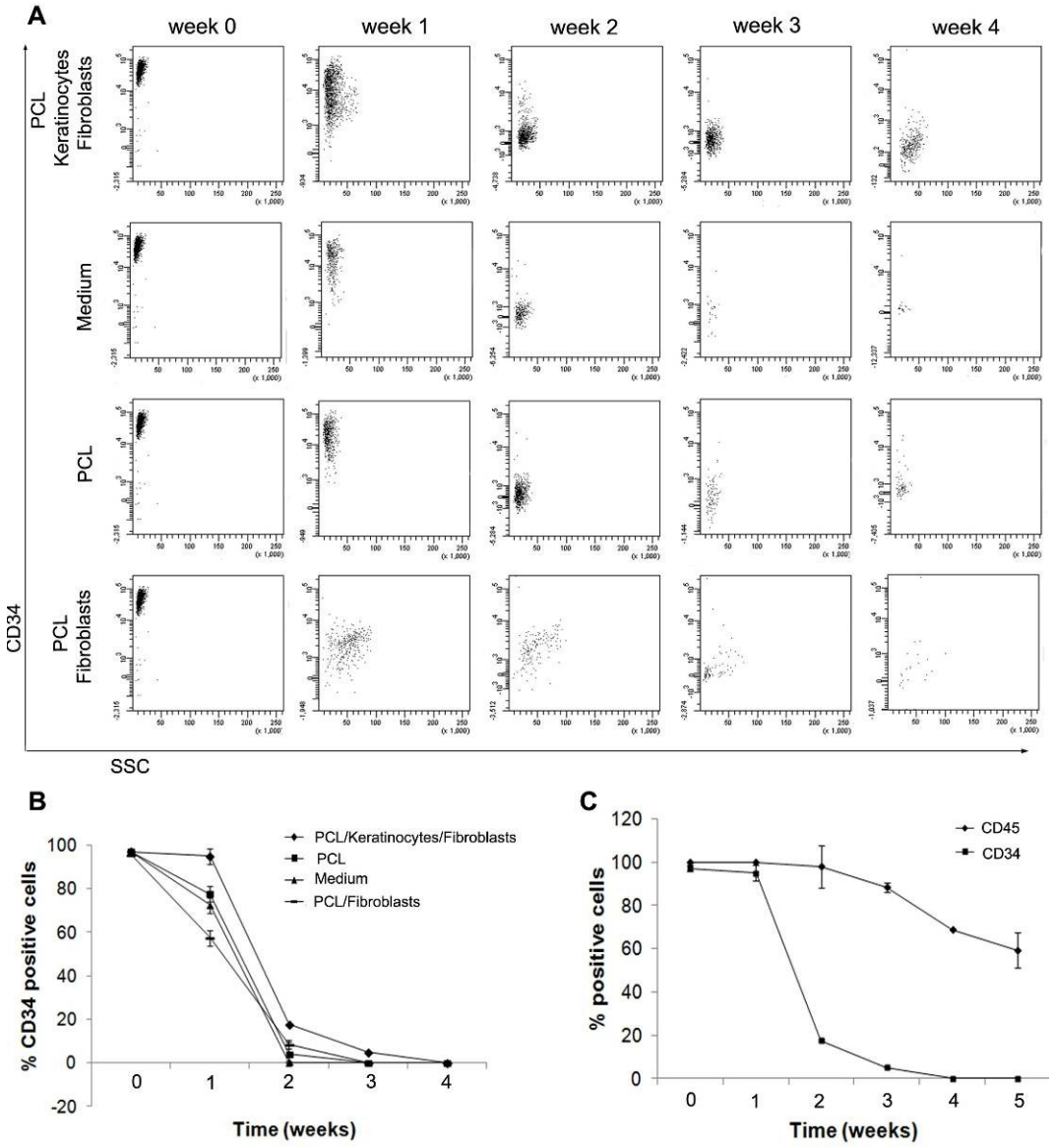

Figure 4. Keratinocytes and fibroblasts seeded on the 3D scaffold support the HSCs survival. Representative flow cytometry data for CD34 (gated on CD45+ cells) cell staining (A) and quantification of CD34+ cells cultured on PCL scaffold/keratinocytes/fibroblasts in comparison to the control systems (B). Measurements were made at 0, 1, 2, 3, and 4 w of culture. Error bars represent the SD of three independent experiments. (C) Behaviour of CD34+ HSCs and CD45+ cells in the PCL scaffold/keratinocytes/fibroblasts system. Measurements were made at 0, 1, 2, 3, 4, and 5 w of culture. Error bars represent the SD of three independent experiments.

We observed that in the multicellular biocomposite, CD7+ cells were de novo generated by the 3rd w of culture. Subsequently, at the 5th w of culture, these

cells also expressed the CD1a marker. Furthermore, CD4 immature single positive cells, not yet expressing CD3 and CD8 markers, were detected during the culture (**Figure 5)**.

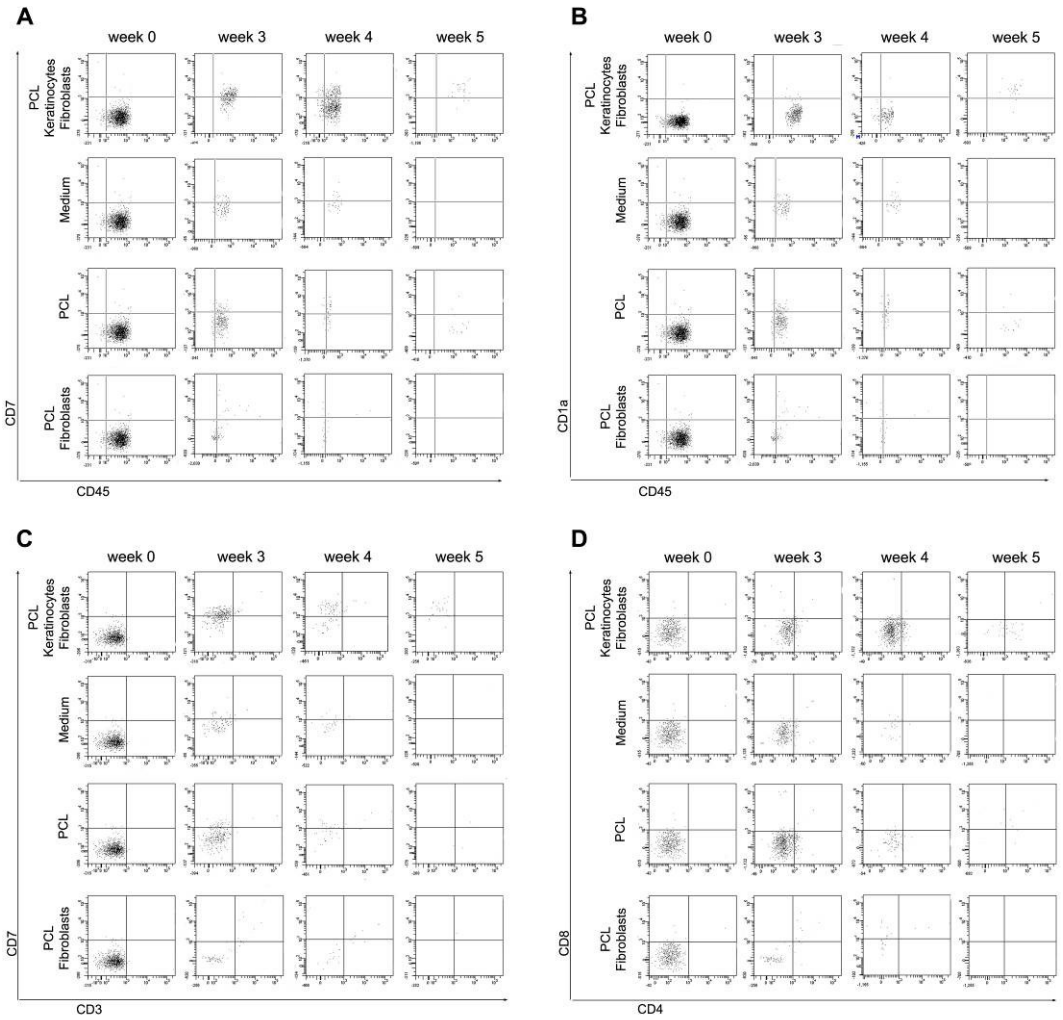

Figure 5. Keratinocytes and fibroblasts seeded on the 3D scaffold support the HSCs differentiation into T-lineage committed cells. (A-B-C-D) Representative flow cytometry data for CD45, CD7, CD1a, CD3, CD4, CD8 cells staining of the cells cultured on PCL scaffold/keratinocytes/fibroblasts in comparison to the control systems at different time points. Measurements were made at 0, 1, 2, 3, 4, and 5 w of culture. Results shown are representative of at least three independent experiments.

These findings are in keeping with the observation that thymic precursors, at the earliest stages of T-cell development, first acquire the CD7 marker [68]. However, these cells along with T cells can also give rise to NK and myeloid

precursors [69]. The transition to $CD7^{+}CD1a^{+}$ stage is considered peculiar of a T-cell commitment. At the next developmental stage, the pre-T cells express CD4, but not yet CD3 and CD8, and are thought to be CD4 immature single positive (ISP) cells. Later in the differentiation process, the cells express CD4 and CD8 and are referred to double positive cells, that subsequently mature in single positive CD4 or CD8 cells [70].

In our study, through the evaluation of the expression patterns of genes selectively expressed in the hematopoietic component of the multicellular biocomposite, we confirmed that an in vitro de novo generation of cells committed toward the T-cell lineage occurred. Of note, TAL1 was down-regulated and Spi-B up-regulated in the cell suspension, consistently with the loss of the multilineage differentiative potential. Moreover, PTCRA and RAG2 expression was detectable at the 3rd w, indicative of a recombinant activity (**Figure 6**). These molecular events were not observed in the control systems and are consistent with the immunophenotypic data, supporting an ongoing T-cell differentiation process.

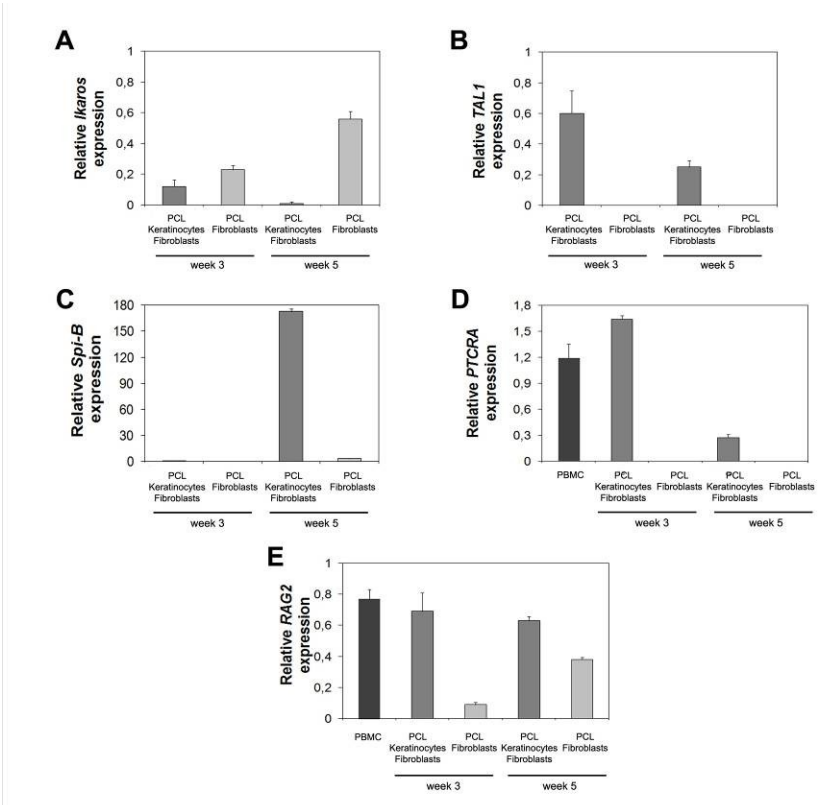

Figure 6. Expression patterns of selected T-lineage specific genes during the HSCs differentiation in the multicellular biocomposite. Real-time PCR evaluation of Ikaros (A), TAL1 (B), Spi-B (C), PTCRA (D) and RAG2 (E) expression in suspensions of hematopoietic cells grown on PCL scaffold/keratinocytes/fibroblasts or control systems. Gene expression was normalized to the expression of β-actin. The values indicate the relative mRNA expression. Data represent the mean ± SD from two independent experiments.

It is known that at early stages of this process, the cells initially express TAL1, which sustains the lineage plasticity. These cells, therefore, retain the potential to become other hematopoietic cell types [71,72]. The down-regulation of this molecule is strongly correlated with the activation of T-cell gene expression program, whose hallmark is the expression of Spi-B and the genes involved in the TCR rearrangement, such as RAG1/2 and PTCRA [73]. Eventually, the cells are committed to a T-cell fate [74].

Our data, indicating that keratynocytes are able to sustain the process, are not surprising, since epithelial and stromal cells of the thymus and skin derived keratinocytes share a remarkable number of similarities. Of note, keratinocytes express FOXN1, a developmentally regulated transcription factor, selectively expressed in epithelial cells of the thymus and skin, where it plays a critical role in cell differentiation and survival resulting in T lymphopoiesis [33,49]. FOXN1 is also expressed in all TECs during initial thymus organogenesis and is required for the initial phase of their differentiation [31,37,75]. Genetic alterations of FOXN1 lead to athymia[45,76] and result in humans in a Severe Combined Immunodeficiency phenotype associated with skin annexa abnormalities, referred as the human equivalent of the mice Nude/SCID syndrome [43,46,77]. This athymic condition is more severe than that observed in the other athymic condition represented by the DiGeorge syndrome, which is only characterized by a moderate reduction of T cells, which are under several aspects functional [78]. DiGeorge patients, differently from Nude/SCID ones, have circulating naïve cells, thus suggesting that ectopic thymus anlage or additional structures may contribute to lymphopoiesis. Our data lead us to argue that skin and, in particular, keratinocytes may play such a role thanks to the FOXN1 expression. Of note,

prenatal alteration of the FOXN1 gene in humans prevents the development of the T-cell compartment, in particular leading to a complete blockage of the CD4+ T-cell lineage maturation [79].

In conclusion, our results indicate that, in a multicellular biocomposite containing skin derived elements in the absence of thymic stroma, HSCs do start differentiating and that the process is also directed toward a T-cell lineage commitment in the presence of IL-7, IL-15 and Flt3-Ligand. However, the maturation process does not lead to the production of fully mature single positive T cells. This suggests that additional factors or molecular manipulations should be used to reproduce a thymic epithelial cell-like surrogate environment. The in vitro re-build of an environment capable to reproduce tissue features of primary lymphoid organs is of valuable help for future therapeutic strategies of patients affected with congenital hematologic and immunologic disorders.

§1.4 Conclusive remarks

Despite an extensive knowledge about the thymus role to foster T-cell development is available, some still unexplained evidence in human athymic conditions suggests that in depth information of this process is still to be achieved and, in particular, the involvement of different non lymphoid tissues in T-cell ontogeny.

The simultaneous occurrence of severe functional T-cell immunodeficiency and skin abnormalities associated to *FOXN1* alterations indicates that the factor exerts a critical role in the development and homeostasis of these epithelia and suggests shared functions of the gene in both thymus and skin epithelium.

Despite the significant progress, the detailed mechanism by which FOXN1 controls the T-cell differentiation process through intercellular cross-talk still remains to be clarified.

Additional knowledge in this field would be very helpful in conclusively defining the role of FONX1 in the biological process, in clarifying the intimate mechanisms of FOXN1 action and in the development of novel therapeutic strategies for congenital disorders of immune system.

CHAPTER II

"Impaired regulation of the immune system"

Within the thymus, developing thymocytes undergo positive and negative selection, resulting in mature T cells that are able to successfully recognize a wide range of foreign antigens and, at the same time, able to ignore self-antigens. Potentially, autoreactive naive T cells, which succeed in reaching the periphery, are functionally suppressed by several mechanisms, in a process known as peripheral tolerance. Breakdown of the central or peripheral tolerance leads to the development of destructive autoimmune reactions and, paradoxically, in a few circumstances also to immunodeficiency, as seen in experimental conditions regarding alteration of the transcription factor Autoimmune Regulator (AIRE) protein or FOXP3. Recent evidence suggests that systemic autoimmunity and immunodeficiency can be strictly interconnected. In immunodeficiencies, the decreased or abolished capacity of the immune system to clear infections causes a continuous immunological stimulation and activation, which represents one of the potential mechanisms that eventually leads to autoimmunity. Dysregulation of immunoregulatory factors combined to multiple genetic variations is responsible for systemic autoimmunity. In a few cases, along with a predominant autoimmune phenotype, an increased susceptibility to infections is also present [80]. Several studies have reported evidence indicating that autoimmune phenomena occur in patients affected with primary immunodeficiencies, and have focused on the molecular and cellular mechanisms underlying these conditions, in order to elucidate the link between autoimmunity and immunodeficiency. On the other hand, studies on genetic autoimmune disorders are also extremely useful for our understanding of the pathophysiology of the intimate mechanisms that lead autoreactive clones to escape clonal deletion [81,82]. Currently, unique models, which help unravel many aspects of the development, homeostasis and regulatory properties of the immune system, are the monogenic autoimmune disorders and, in particular, autoimmune polyendocrinopathy candidiasis ectodermal dystrophy

(APECED), immunodysregulation, polyendocrinopathy and enteropathy X-linked (IPEX), autoimmune lymphoproliferative syndrome and IL-2 receptor α-chain (IL-2RA, CD25) deficiency. Impairment of central or peripheral tolerance, which normally prevents the survival, expansion and activation of autoreactive T cells, thus protecting from autoimmune diseases, is the main pathogenetic mechanism responsible for these conditions.

§2.1 Abnormalities of the central and peripheral tolerance

Self–nonself discrimination plays a key role in inducing a productive immunity and in preventing autoimmune reactions. Altering this balance will result in immunodeficiency or autoimmunity. In this context, positive and negative selections are crucial for the creation of a T-cell repertoire able to respond to a huge number of foreign antigens, preserving, at the same time, the tolerance to self-antigens expressed in the various tissues [83].

Tolerance represents a state of immunologic nonresponsiveness in the presence of a particular antigen. The immune system is able to discriminate between self- and nonself-antigens. This property is fundamental to induce a productive immunity and to prevent autoimmune reactions. Altering this balance will result in immunodeficiency or autoimmunity. Immune tolerance to self-antigens is acquired through two main processes: central and peripheral tolerance [83]. Central tolerance takes place within the thymus through the negative selection process, which occurs subsequently to the positive one. Eventually, the tolerance to self-antigens expressed in various tissues is acquired [83].

Within the thymic medulla, thymic medullary epithelial cells (mTECs) and medullary dendritic cells (mDCs) play a central role in the establishment of self-tolerance through the negative selection process [84]. Both cellular types, expressing the costimulatory molecules, CD40, CD80, CD86 and MHC class II, are able to favor an efficient thymocyte deletion. mTECs express a thousand genes, the so-called ‘promiscuous gene expression’, including tissue-specific self-antigens (TSAs), normally present only in specialized peripheral organs [85]. This high expression by mTECs of genes encoding TSAs is driven by transcription

factor autoimmune regulator (AIRE) [86-88]. These MHC-restricted self-antigens are presented by mTECs to developing T cells. Thymocytes that recognize self-antigens with high affinity/avidity are deleted [89]. Mutations of AIRE result in autoimmune polyendocrinopathy candidiasis ectodermal dystrophy, a rare autosomal recessive disease (OMIM 240300), which is the paradigm of a genetically determined failure of central tolerance and autoimmunity [90,91].

Some of the thymocytes, which bind self-MHC–peptide complexes with high affinity, express Foxp3, and through a yet unknown mechanism escape negative selection and differentiate into Tregs, which play an important role in the peripheral tolerance suppressing autoreactive T cells in the periphery [92,93]. Triggering the costimulatory molecules and the subsequent activation of Fas signaling pathway represents the commonly accepted mechanism to explain the deletion of self-reactive thymocytes within the thymus [94].

Fas-Fas Ligand interactions are implicated in the elimination of self-reactive T cells by a process known as restimulation induced cell death. The role of Fas is, thus, restricted to the elimination of T cells specific for autoantigens and chronic pathogens. This process has a crucial role in maintaining self-tolerance in T cells that have escaped central thymic tolerance. T-cell receptor (TCR) engagement of activated T cells results in FasL gene upregulation and secretion [95] and in translocation of Fas to lipid rafts providing a critical and physiologically significant signal that sensitizes T cells to Fas-mediated apoptosis. FasL is constitutively expressed on cells of immune privileged organs, such as brain, anterior chamber of eyes, and testes. In consequence, FasL protects these privileged sites from the action of immune system cells, as an additional regulatory mechanism of self-tolerance. On the contrary, FasL expression is usually transient on activated T cells. Many signaling pathways and transcription factors mediates inducible FasL expression T cells. Fas is also implicated in B cell function regulating autoantibody production and antigen presenting cell function. In particular Fas mediates peripheral B cell tolerance.

Mutations affecting Fas and FasL confer potent susceptibility to autoimmune diseases, such as Autoimmune lymphoproliferative syndrome (ALPS).

§2.1.1 Altered central tolerance and autoimmune disease

The paradigm of a genetically determined failure of central tolerance and autoimmunty is the APECED, a rare autosomal recessive disease due to the mutation of the AIRE gene. Patients with APECED have a highly variable pattern of destructive autoimmune reactions toward different endocrine and nonendocrine organs mainly involving parathyroid glands, adrenal cortex, gonads, pancreatic beta cells, gastric parietal cells, and thyroid gland. Moreover, ectodermal abnormalities are frequently present. The classical clinical diagnosis is primarily based on the presence of two of the three most common clinical features: chronic mucocutaneous candidiasis (CMC), chronic hypoparathyroidism (CH), and Addison disease (AD) [96]. The presence of only one component is sufficient for the diagnosis if a sibling is affected [97]. However, although APECED is a monogenic disorder, it is characterized by a wide variability of the clinical expression, thus implying a further role for disease-modifying genes and environmental factors in the pathogenesis.

We also described a patient in which adrenal failure was the presenting feature of primary primary antiphospholipid syndrome (APS).

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Genetic Basis of Altered Central Tolerance and Autoimmune Diseases: A Lesson from AIRE Mutations

Donatella Capalbo, MD, PhD, Giuliana Giardino, MD, Lucia De Martino, MD, Loredana Palamaro, PhD, Rosa Romano, PhD, Vera Gallo, MD, Emilia Cirillo, MD, Mariacarolina Salerno, MD, PhD, and Claudio Pignata, MD, PhD

Department of Pediatrics, "Federico II" University, Naples, Italy

The thymus is a specialized organ that provides an inductive environment for the development of T cells from multipotent hematopoietic progenitors. Self–nonself discrimination plays a key role in inducing a productive immunity and in preventing autoimmune reactions. Tolerance represents a state of immunologic nonresponsiveness in the presence of a particular antigen. The immune system becomes tolerant to self-antigens through the two main processes, central and peripheral tolerance. Central tolerance takes place within the thymus and represents the mechanism by which T cells binding with high avidity self-antigens, which are potentially autoreactive, are eliminated through so-called negative selection. This process is mostly mediated by medullary thymic epithelia cells (mTECs) and medullary dendritic cells (DCs). A remarkable event in the process is the expression of tissue-specific antigens (TSA) by mTECs driven by the transcription factor autoimmune regulator (AIRE). Mutations in this gene result in autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED), a rare autosomal recessive disease (OMIM 240300). Thus far, this syndrome is the paradigm of a genetically determined failure of central tolerance and autoimmunty. Patients with APECED have a variable pattern of autoimmune reactions, involving different endocrine and nonendocrine organs. However, although APECED is a monogenic disorder, it is characterized by a wide variability of the clinical expression, thus implying a further role for disease-modifying genes and environmental factors in the pathogenesis. Studies on this polyreactive autoimmune syndrome contributed enormously to unraveling several issues of the molecular basis of autoimmunity. This review focuses on the developmental, functional, and molecular events governing central tolerance and on the clinical implication of its failure.

Keywords AIRE, autoimmune diseases, autoimmunity, central tolerance, susceptibility genes

Recent evidence suggests that systemic autoimmunity and immunodeficiency can be strictly linked. One of the mechanisms is related to the decreased ability of immune system to clear the infections in patients with immunodeficiencies, which cause perpetual immune-system activation and, eventually, autoimmunity. Systemic autoimmunity is due to combined effect of multiple genetic variations, infections, and immunoregulatory factors that, along with a predominant autoimmune phenotype, may also lead to an increased susceptibility to infections. In the light of this novel point of view, the overlap of clinical manifestations suggests that immunodeficiency should be considered in the presence of autoimmunity and vice versa [1].

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Address correspondence to Claudio Pignata, MD, PhD, Professor of Pediatrics, Department of Pediatrics, Unit of Immunology, "Federico II" University, via S. Pansini 5-80131, Naples, Italy.

Growing evidence has been accumulated indicating that autoimmune phenomena occur in patients suffering from primary immunodeficiencies (PID), and the molecular and cellular mechanisms that interconnect these conditions begin to be elucidated. The study of rare single-gene disorders associated with significant autoimmunity certainly and greatly contributed to the overall comprehension of the pathophysiology of the complex and intimate mechanisms underlying autoimmune disorders [2].

In this regard, monogenic autoimmune diseases, such as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED), immunodysregulation, polyendocrinopathy and enteropathy X-linked (IPEX), autoimmune lymphoproliferative syndrome (ALPS), and interleukin (IL) 2 receptor α-chain (IL-2RA, CD25) deficiency, offer a unique model to unravel many aspects of the development, homeostasis, and function of the immune system as well as of the balance between autoimmunity and immunodeficiency. Under these conditions, central or peripheral tolerance, which normally prevents the survival, expansion, and activation of autoreactive T cells, thus protecting the system from autoimmune diseases, are impaired.

As a paradigm of abnormal peripheral tolerance, ALPS is a disorder characterized by nonmalignant lymphoproliferation, increased risk of lymphoma, and autoimmunity, often manifesting as multilineage cytopenias [3, 4]. The most common genetic alterations are heterozygous germline mutations in the gene encoding the TNF receptor-family member Fas (CD 95, Apo-1) [5–9], somatic Fas mutations and mutations in the genes encoding Fas-ligand (*FASLG*), caspase 10 (*CASP10*) and caspase 8 (*CASP8*), and NRAS and KRAS [10, 11].

IPEX is a further example of failure of the peripheral tolerance, characterized by immune dysfunction, polyendocrinopathy, enteropathy, and X-linked inheritance [12, 13]. This syndrome is caused by mutations in the forkhead box P3 (*FOXP3*) gene located in the short arm of chromosome X (Xq11.23-Xq13.3). This gene consists of 11 exons, which encode a protein of 431 amino acids, and serves as a lineage specification factor of regulatory T lymphocytes (Tregs) [14–16]. FOXP3 plays a crucial role in the generation of Tregs. Tregs are a thymus-derived cell subset [17], which plays a central role in the regulation of immune responses to self-antigens, allergens, and commensal microbiota as well as immune responses to infectious agents and tumors. A defective Treg function is associated with autoimmunity, allergy, and immunodeficiency [18, 19]. In contrast, an increased Treg function has been associated with malignancies [20–23].

In a few IPEX-like cases, alterations of other molecules involved in the generation of Tregs, such as the IL-2RA, have been reported [24, 25].

Although ALPS, IPEX, and IL-2RA deficiency are examples of genetic alterations that greatly contributed to a better understanding of the role of peripheral tolerance mechanisms, most of the current knowledge on the pathogenesis of autoimmunity also arises from the discovery of genetic models of abnormal central tolerance. In this review, we focus our attention on the paradigmatic genetic alterations resulting in the failure of central tolerance.

THYMIC DEVELOPMENT AND INTRATHYMIC SELECTION

The thymus is a specialized organ that provides an inductive environment for the development of T cells from multipotent hematopoietic progenitors [26–28] and T-cell repertoire selection, which promotes differentiation of thymocytes expressing T-cell receptors (TCR) with intermediate affinity and/or avidity for self-peptide-MHC complexes and allows intrathymic removal of thymocytes that express TCR with high

affinity for self-antigens. These two processes, respectively known as positive and negative selection, are required to ensure a high degree of self-tolerance.

On entering the thymus, immature thymocytes promote the differentiation of precursor thymic epithelial cells (pTECs) into cortical TECs (cTECs) and medullary TECs (mTECs), playing an important role in the formation of the thymic microenvironment [29–31]. The passage of thymocytes through discrete thymic microenvironments together with the interaction with different types of stromal cells and antigen presenting cells (APCs) has an important role in the survival and fate choices of developing T cells.

On the basis of the different immunophenotypic patterns, T-cell development can be divided into three subsequent steps: the $CD4^{-}CD8^{-}$ DN stage, the $CD4^{+}CD8^{+}$ double-positive (DP) stage, and the $CD4^{-}CD8^{+}$ or $CD4^{+}CD8^{-}$ single-positive (SP) stage (Figure 1A). The DN1 thymocytes are multipotent cells in that they may still differentiate into B, T, myeloid, natural killer (NK), and dendritic cells (DCs) [32–34]. The DN2 thymocytes lose the multilineage potential even though, under certain circumstances, they can still differentiate into NK cells, DCs, or macrophages [35, 36]. DN2 to DN3 transition requires the expression of a different array of genes, which allows full TCRβ gene rearrangement in thymocytes, which become competent to undergo β-selection [37, 38]. The induction of recombinase activating gene-1 (*RAG-1*) and

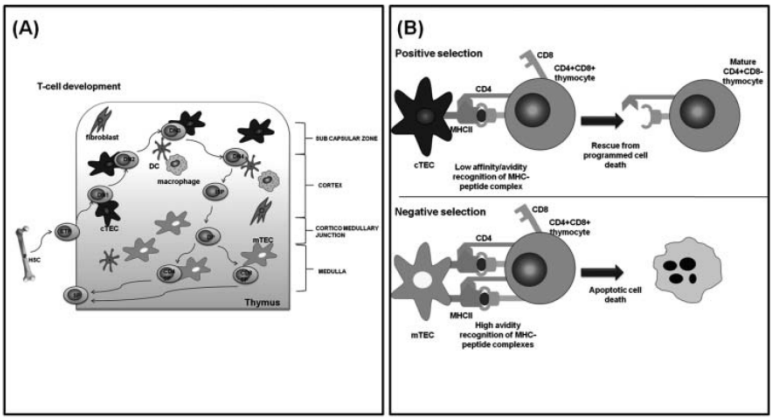

FIGURE 1. Thymic development and intrathymic selection. (A) The thymus is a specialized organ that provides an inductive environment for the development of T cells from multipotent hematopoietic progenitors. The intrathymic development of T cells consists of several phases that require a dynamic relocation of developing lymphocytes within multiple architectural structures of this organ. These steps are the entry of lymphoid progenitor cells into the thymus; the generation of $CD4^{+}CD8^{+}$ double positive (DP) thymocytes in the cortex; the positive selection of DP thymocytes in the cortex and the interaction of positively selected thymocytes with medullary thymic epithelial cells (mTECs) to complete the thymocyte maturation; and, eventually, the export of mature T cells from the thymus. (B) Positive and negative selections are crucial for the creation of a T-cell repertoire able to respond to a huge number of foreign antigens, preserving, at the same time, the tolerance to self-antigens expressed in the various tissues. During positive selection (top panel) DP thymocytes, which do not get a rescue signal through TCR, are programmed to undergo "death by neglect" or apoptosis. Within the thymic medulla, negative selection (bottom panel) is mediated by mTECs and medullary DCs. The prominent process in inducing the negative selection is represented by the broad expression of tissue-specific antigens (TSA). T cells binding self-antigens with high avidity, potentially autoreactive lymphocytes, are eliminated through the negative selection process.

RAG-2, the upregulation of pre-Tα ($pT\alpha$), and the rearrangement of TCRδ and γ also occur during the DN2/3 transition. T-cell precursors in this stage lose their capability to follow a non-T-cell fate choice [39]. The cells overcoming β-selection express the pre-TCR complex on their surface and reach the DN3 stage [40]. Pre-TCR signaling downregulates *CD25*, $pT\alpha$, *RAG-1,* and *RAG-2*, leading to the DN4 stage. DN4 cells are fully committed to the $\alpha\beta$ T-cell lineage [41, 42].

After β-selection, the thymocytes, which have properly rearranged TCRβ chains, show a burst of proliferation and a subsequent upregulation of CD8 and then CD4. At this point the cells become DP. Eventually, DP cells rearrange *TCR*α gene, leading to TCRα assembly into a TCR complex. This DP population, with an unselected repertoire, must undergo the positive selection [43, 44] to continue intrathymic development. DP thymocytes positively selected are induced to differentiate into SP cells and to migrate into medulla, where self-reactive thymocytes are deleted through negative selection.

Thereafter, SP thymocytes, with an appropriate TCR repertoire, leave the thymus as recent thymic emigrants (RTE), naïve cells expressing the CD62 ligand (CD62L), CD69, and the CD45RA isoform. These RTE cells are fully mature T cells that exert proper functional capabilities of cell-mediated immunity [45–47].

POSITIVE AND NEGATIVE SELECTION

Self–nonself discrimination plays a key role in inducing a productive immunity and in preventing autoimmune reactions. Altering this balance will result in immunodeficiency or autoimmunity. In this context, positive and negative selections are crucial for the creation of a T-cell repertoire able to respond to a huge number of foreign antigens, preserving, at the same time, the tolerance to self-antigens expressed in the various tissues [48].

DP thymocytes, in order to increase the ability to get through positive selection, rearrange the TCRα locus in a sequential manner, during a process referred to as receptor editing. After 3 days, DP thymocytes, which do not get a rescue signal through TCR, are programmed to undergo "death by neglect" or apoptosis (Figure 1B). Only about 5% of DP is able to bind a MHC ligand with mild avidity getting a signal that induces DP maturation to the $CD4^+$ $CD8^-$ or $CD4^-CD8^+$ SP stage [49–51] and triggers the expression of chemokines that direct the T cell through the subsequent maturation step within the thymic medulla [52, 53].

Tolerance represents a state of immunologic nonresponsiveness in the presence of a particular antigen. The intrathymic central tolerance is the mechanism by which T cells binding self-antigens with high avidity, potentially autoreactive lymphocytes, are eliminated through the negative selection process (Figure 1B) [48, 54–57]. Autoreactive lymphocytes that have escaped negative selection are deleted in the periphery through the mechanisms of the peripheral tolerance [48, 58].

Within the thymic medulla negative selection is mediated by mTECs and medullary DCs (mDCs). Mature mTECs display a peculiar phenotype, characterized by the high surface expression of CD40, CD80, CD86, and MHC class II, which favors an efficient thymocyte deletion. The prominent process in inducing the negative selection is represented by the broad expression of tissue-specific antigens (TSA) [59-62]. This high expression by mTECs of genes encoding TSA, normally found in the periphery, is driven by the transcription factor autoimmune regulator (AIRE) [59, 63–67]. These TSA include, among other tissues, proteins restricted to the pancreas, stomach, eye, salivary gland, muscle, and thyroid [59, 63, 68–70]. However, AIRE doesn't regulate the expression of all TSA genes by mTECs. Surprisingly, only 1–2% of mTECs express a given TSA, making it very difficult to understand how a low number of mTECs is able to delete a

so huge number of clonotypes [65, 67, 71]. It has been hypothesized that during a 4- to 5-day period the high motility of thymocytes allows them to make a huge number of interactions with mTECs [72, 73]. mDCs have a similar role, even though they acquire TSA mostly through the uptake of apoptotic mTECs. Alternatively, they migrate from the periphery into the thymus [74–76].

Some of the thymocytes that recognize self-MHC-peptide complexes with high affinity express Foxp3 and mature as Tregs, which are able to suppress autoreactive T cells in the periphery [64, 77–79]. To mature into Tregs, the precursor cell must bind self-antigens with high avidity [80]. In the mouse model, it has been shown that strong and not weak interactions between TCR and agonist peptide lead to the development of Foxp3$^+$ Tregs with regulatory properties [81–83]. However, there is evidence suggesting the involvement of additional factors in the natural differentiation of Tregs [84].

Of note, two mechanisms to suppress self-reactive thymocytes have been proposed: triggering of co-stimulatory molecules and the activation of Fas signaling pathway. In particular, it has been shown that the co-stimulatory molecules such as CD40, CD80, and CD86, which are expressed by mTECs and mDCs, play a pivotal role in clonal deletion [85, 86]. Indeed, exposure of DP thymocytes to anti-TCR monoclonal antibody (mAb) fails to induce thymocytes death, but not in the presence of APCs [87]. In the presence of APCs expressing B7 molecules, a crucial co-stimulatory factor, which interacts with thymocyte CD28 receptor, DP thymocytes undergo apoptosis using anti-TCR and anti-CD28 mAb in vitro [88]. Furthermore, it has been observed in SP thymocyte cultures that the exposure to high concentration of anti-TCR mAb is able to induce apoptosis through a high-level TCR signaling in the absence of co-stimulation [88]. This effect is abolished [89] in Fas-lpr/lpr cells [90], carrying homozygous mutation of Fas, a member of the tumor necrosis factor receptor superfamily. Thus, the strong TCR ligation leads to apoptosis in a Fas-dependent manner in the absence of co-stimulatory signals [89, 90]. On the contrary, moderate-avidity TCR binding requires interactions with co-stimulatory molecules to induce apoptosis and, therefore, under these circumstances, cell death is induced by a Fas-independent mechanism.

MOLECULAR BASIS OF THE CENTRAL TOLERANCE

Mouse and Human Models of Impaired Central Tolerance

To explain the huge number of tissue-specific proteins detected in the thymus, many studies have been till now conducted, in both mice and humans, to unravel the identity of peripheral self-antigen-producing cells within the thymus. Recent reports have shown that both in humans and mice, self-antigen expression in the thymus is restricted to mTECs [59, 91] and, in particular, to mTECs forming the Hassal corpuscle (HC). The molecular mechanisms governing self-antigen expression by mTECs of HC and the function of HC in the negative selection process remains to be clarified. Studies in human thymus show that mTECs in HC express CD30-L, a membrane-associated glycoprotein involved in T-cell signaling. Interestingly, CD30-deficient mice show a gross defect in negative but not positive T-cell selection [92].

Studies have shown that a reduced expression of a specific TSA by mTECs may be associated with the development of that specific autoimmune disorder. For example, susceptibility to type 1 diabetes (T1D) in humans has been strongly linked to polymorphisms in variable number of tandem repeats (VNTRs) within insulin promoter, which correlate with the level of mRNA expression within the thymus. A reduced thymic insulin expression may reduce the efficacy of clonal deletion promoting an increased escape of insulin-specific T cells. In addition, the variability of insulin expression levels in the thymus, when compared to the pancreas, also implies that insulin expression is regulated differently in these two organs [93]. Low expression of insulin in the

thymus of nonobese diabetic (NOD) mice has also been reported [94] and may play a role in diabetes susceptibility in this mouse strain. A point mutation in the *CHRNA1* gene, encoding the α-subunit of the muscle acetylcholine receptor has recently been described in a subset of patients with myasthenia gravis. These mutations are able to prevent the interferon (IFN) regulatory factor 8 binding to the *CHRNA1* gene, reducing its transcription in mTECs [95].

Negative selection of autoreactive thymocytes may also be impaired by alterations in TSA-specific mRNA processing. For example, in the mouse model of autoimmune encephalomyelitis, mRNA splicing deletes the expression of proteolipid protein peptide-specific thymocytes, thus leading to autoimmunity [96, 97].

Genetic alterations of thymocytes may also lead to autoreactive T-cell escape. It is well documented that the strength of signaling transduced by TCR upon binding of self-peptide MHC complex plays a key role in clonal deletion. For example, SKG mice, characterized by a point mutation in the C-terminus of the SH2-domain of ZAP-70, develop rheumatoid arthritis (RA)-like disease. Mutations in ZAP-70, in fact, result in reduced TCR signaling, thus limiting the deletion of the arthritogenic thymocytes [98]. A reduced thymic negative selection may also derive from a dysregulation of the pathways inducing apoptosis in the thymocytes. For example Nur77 is a pro-apoptotic molecule implicated in the thymocyte negative selection. In Nur77-deficient mice, autoreactive thymocytes are resistant to apoptosis, and reduced expression of Nur77 has been reported in a number of autoimmune disease [99].

Aberrant development of mTECs may also impact the efficacy of negative selection. For example, AIRE expression in the thymus requires cross-talk between developing thymocytes and stromal cells, and this process is dependent on an organized thymic microenvironment [100, 101]. A higher incidence of autoimmune phenomena, including autoimmune cytopenias [102, 103] autoimmune arthritis [104], and autoimmune endocrinopathies [105], can be observed in DiGeorge syndrome (DGS) [106, 107] in which abnormal thymic development may result in impaired expression of AIRE and, potentially, of other transcription factors that regulate expression of organ-specific antigens in the thymus resulting in defective central tolerance [100, 108]. However, it should be mentioned that in DGS autoimmunity may also be explained by exaggerated chronic inflammatory responses, "bystander" activation of autoreactive T cells and "molecular mimicry" [109]. Thymic abnormality in DGS may also impair the generation of Tregs. Evidence is available showing that patients with DGS have significantly lower Treg counts compared with healthy controls [110]. The decreased counts and proportions of Tregs in patients with developmental thymic hypoplasia suggest that the generation and maintenance of the FoxP3 Treg pool, at least in children, is directly related to thymic function rather than peripheral production [110].

Finally, a paradigmatic example of genetic alteration of central tolerance is the mutation of the *AIRE* gene. The *AIRE* gene maps in humans to chromosome 21q22.3, and was cloned in 1997 by two independent research groups [111, 112]. It consists of 14 exons spanning 11.9 kb of genomic DNA [113] and encodes a 58-kDa protein [111].

Three different isoforms of AIRE protein can be generated by alternative splicing. However, a unique combination of domains, common to transcriptional regulators and chromatin-binding proteins, has been identified. The N-terminal region shows a 6-helix structure with high similarity to a caspase-recruitment domain (CARD) [114]. This CARD domain is required for oligomerization of AIRE [115, 116]. Moreover, AIRE contains an Sp100, AIRE1, NucP41/75, DEAF1 (SAND) domain [117] important for AIRE transactivation capacity and subcellular localization [118]. The C-terminal region contains 2 plant homeodomain PHD- type zinc fingers [119]. Mutations in the PHD domains lead to a severe decrease of AIRE transcriptional activation capacity [120–122]. In particular, disease-causing missense mutations in PHD1 domain have

been shown to abolish E3 ligase activity [123]. Other structural features include a conserved nuclear localization signal and 4 LXXLL motifs or nuclear receptor interaction domains [124]. AIRE protein is mainly localized in the cell nucleus, within nuclear bodies distributed near to nuclear speckles [120], suggesting the involvement of AIRE in the regulation of gene transcription.

The precise molecular mechanisms underlying AIRE function remain to be clarified. The large number and chromosomal clustering [125] of the AIRE-regulated gene suggest that it does not act as a conventional DNA-binding transcription factor, but as a coactivator in a large transcriptional complex. Moreover, recent studies on human embryonic kidney 293T cells have demonstrated that AIRE interacts with a large set of binding partners [126]. The proteins could be divided into 4 major functional classes: nuclear transport, chromatin binding/structure, transcription, and pre-mRNA processing [126]. The first protein reported to bind to AIRE was CREB-binding protein (CBP) [127]. CBP is important for transcription initiation and histone and nonhistone protein acetylation. Its interactions with AIRE may lead to promotion of gene transcription through histone acetylation and the recruitment of chromatin-transcription factors [115, 128, 129]. Other AIRE partners have been identified, such as DNA protein kinase (DNA-PK) and SP-RING domain protein inhibitor of activated STAT1 (PIAS1) (Figure 2) [130, 131]. DNA-PK deficiency in the stromal compartment leads to decreased TSA expression in mTECs, thus resulting in autoantibody production [122, 130]. AIRE also binds and recruits the positive transcription elongation factor b (P-TEFb) complex to RNA polymerase II, eventually targeting gene promoters and enhancing the transcription-elongation process [132]. The ability of AIRE's PHD1 finger domain to bind histone 3 molecules with unmethylated lysine at position 4, generally associated with repressed genes, indicates a possible epigenetic control of the AIRE target genes [133, 134]. It is plausible that AIRE recruitment of all its co-transcriptional partners to TSA gene promoters activates the expression of the large number of TSA in mTECs [135].

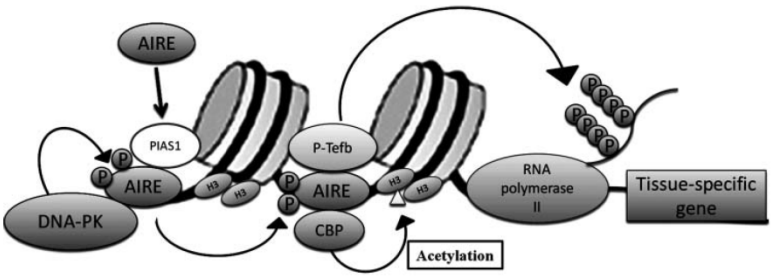

FIGURE 2. AIRE's PHD1 finger domain is preferentially recruited to histone 3 molecules with unmethylated lysine at position 4, indicating a possible epigenetic control of the AIRE target genes. On target gene regulatory regions, AIRE recruits a number of partners, which could be divided into 4 major functional classes: nuclear transport, chromatin binding/structure, transcription, and pre-mRNA processing. AIRE binds and recruits the positive transcription elongation factor b (P-TEFb) complex to RNA polymerase II enhancing the transcription-elongation process. AIRE also interacts with CREB-binding protein (CBP), leading to promotion of gene transcription through histone acetylation and recruitment of chromatin-transcription factors. AIRE may also interact with the DNA-dependent protein kinase (DNA-PK) complex and protein inhibitor of activated STAT1 (PIAS1). DNA-PK phosphorylates AIRE and collaborates with AIRE in the formation of chromatin loops. PIAS1 is involved in the nuclear organization of chromatin.

AIRE has also been detected in peripheral lymphoid tissues, such as lymph nodes and spleen, and in other tissues, though at a lower degree than in thymic stromal cells. It is likely that in the periphery, AIRE also contributes to the process of immune tolerance by inducing TSA's gene expression [136–139].

The murine *Aire* gene has been mapped to chromosome 10, revealing a structural organization and sequence homology to its human ortholog [140, 141]. Animal models of $Aire^{-/-}$ have been an important tool in furthering our understanding of how *Aire* prevents autoimmunity. $Aire^{-/-}$ mice present several autoimmune manifestations, mononuclear infiltrates in multiple organs, and the presence of autoantibodies against several tissues [63, 116, 142]. Differently from the human counterpart, $Aire^{-/-}$ mice do not develop *Candida* infections [143].

Along with the central tolerance network, several other peripheral mechanisms are capable of contributing to the control and regulation of the immune system and, presumably, to the clinical expression of the disease. An additional mechanism involved in controlling the reactivity to self-antigens in the periphery is also represented by NK-cell activity. Studies show that resting or cytokine-induced NK cells are able to inhibit activation and/or proliferation of autoreactive clones, as well [144–147].

An example of the association of AIRE alterations with immunodeficiency and autoimmunity is Omenn syndrome [100, 148]. AIRE expression in the thymi of 2 Omenn syndrome patients and 1 $T^{-}B^{-}NK^{+}$ shows profound reduction of AIRE mRNA and protein compared to a normal control subject. Moreover, there was no detectable mRNA for the self-antigens insulin, cytochrome P450 1A2, or fatty acid-binding protein in the immunodeficient patients. The authors concluded that deficiency of AIRE expression occurs in severe immunodeficiencies characterized by abnormal T-cell development and suggested that in Omenn syndrome, the few residual T-cell clones that develop may escape negative selection and thereafter expand in the periphery, causing massive autoimmune reactions [100].

The Human Phenotype Associated with AIRE Gene Mutations: APECED

APECED is a rare disease (OMIM 240300) [111, 112] with a complex clinical phenotype discovered over decades [149]. Patients with APECED have a highly variable pattern of destructive autoimmune reactions toward different endocrine and nonendocrine organs mainly involving parathyroid glands, adrenal cortex, gonads, pancreatic beta cells, gastric parietal cells, and thyroid gland. Moreover, ectodermal abnormalities are frequently present. The main ectodermal manifestations in APECED are dental enamel hypoplasia, pitted nail dystrophy, and alopecia. Keratopathy, vitiligo, and calcification of the tympanic membranes can also be described. Furthermore, autoimmunity in APECED may involve the gastrointestinal system, leading to autoimmune gastritis, malabsorption, and autoimmune hepatitis [150]. The classical clinical diagnosis is primarily based on the presence of two of the three most common clinical features: chronic mucocutaneous candidiasis (CMC), chronic hypoparathyroidism (CH), and Addison disease (AD) [151]. The presence of only one component is sufficient for the diagnosis if a sibling is affected [152]. Autoantibodies for type 1 IFN (IFN-ω and IFN-α) may be considered as a specific and sensitive diagnostic tool for APECED [121, 153, 154]. The APECED varies in the severity and number of disease components. In most patients, CMC precedes the other immune disorders appearing by the age of 5 years, usually followed by CH and later by AD [155]. The complete triad develops in up to two-thirds of patients [151, 152]. As mentioned above, further clinical or latent autoimmune endocrine diseases may be associated [151]. The autoimmune manifestations most likely result from destruction of the target organ by cellular and antibody-mediated attack [152]. In particular, autoantibodies to parathyroid, adrenal glands, and type I IFN are hallmarks of APECED [121, 153]. The molecular

basis of the increased susceptibility to CMC in APECED patients is still poorly understood. Recently, a role of the autoantibodies against the Th17-related cytokines IL-22, IL-17A, and IL-17F has been described in the pathogenesis of the CMC [156, 157]. In particular, IL-17F and IL-22 secretion seems to be significantly decreased in response to *Candida albicans* in APECED patients [156]. Life expectancy depends on the severity of the disease. The overall mortality of patients with APESED is high, but it widely varies on the basis of the clinical spectrum. The most dangerous autoimmune manifestations are fulminant necrotizing hepatitis, severe malabsorption, and tubulointerstitial nephritis [158]. Suboptimal hormonal substitution or inadequate management of addisonian crisis may also increase the mortality risk [159]. Furthermore, patients with long-lasting oral candidiasis are at increased risk of esophageal squamous cell carcinoma [160].

Disease-targeted therapy is not currently available and the treatment mainly relies on hormone replacement and caring for clinical symptoms. So far, immunosuppressant therapy has been considered only for potentially fatal disease, such as hepatitis, nephritis, or severe malabsorption [161]. In our series of patients with childhood onset APECED, the clinical spectrum widely varies between patients suffering only occasional episodes of mild oral candidiasis and stable hypoparathyroidism and patients exhibiting complex phenotypes with life-threatening events [162, 163]. Most of them are well controlled with only hormonal replacement therapy. Experimental immunomodulatory treatment of *Aire* knockout mouse targeting T and B cells might open the perspective of a similar strategy also in humans [164, 165].

Although rare, APECED has been reported worldwide [150] with a wide variability in its incidence. The estimated prevalence in some genetically isolated populations is relatively higher (1:9000 in the Iranian Jews [166], 1:25000 in Finns [152, 167], and 1:14.400 in Sardinians [168]. It is also quite frequent in Norway [154] and Italy [162]. Even though the most frequent mode of inheritance is autosomal recessive, an Italian family with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy harbored a missense (G228W) mutation in the exon 6 in heterozygosity, indicating a dominant pattern of inheritance [169].

So far, over 60 different mutations of the *AIRE* gene have been documented in APECED patients [70]. Some different mutations have been found to be peculiar of specific areas. R257X is the most common mutation among Finnish and other European patients [170–172], 1094-1106del113 (or 967-979del13 bp) is the most common mutation in British [173], Irish [174], North America [175, 176], and Norwegian patients [154], and the Y85C mutation is more frequent among Iranian Jews [120]. In Italy APECED shows an increased prevalence in various regions, in particular in Sardinia, Apulia, and the Venetian area. Moreover, both in Sardinia and Apulia peculiar mutations of *AIRE* have been identified: the mutation R139X on exon 3 in Sardinia [177, 178] and the mutation W78R on exon 2 in Apulia [179]. In the Veneto region, *AIRE* mutations (R257X on exon 6 and 8) were different from the other Italian regions but similar to that identified in Finnish and Anglo-Saxon patients [180]. A typical mutation has been recently identified also in Sicily (R203X on exon 5) [181, 182]. The patients from Campania showed high frequency of mutations in the region of exon/intron 1 [162]. No AIRE gene mutations specific to Calabria have been found in patients with APECED. Only mutations similar to those found in patients from Apulia and Sicily were identified [183].

As mentioned above, although APECED is a monogenic disorder, it is characterized by a wide variability in clinical expression, each patient showing a different pattern of affected organs and autoantibody specificities. In the largest reported series of 91 Finnish patients, a wide variation of the clinical phenotype and course of APECED has been documented [150]. Later on, many other authors confirmed this phenotypic

heterogeneity among several populations [162, 167, 168, 171, 174, 176, 180, 184] but a precise genotype–phenotype correlation is still lacking. Of note, the clinical features vary not only among patients from different families but also among siblings carrying the same mutation [162]. Such variability strongly suggests that disease-modifying genes, environmental factors, as well as immune system dynamics may play a role in modulating clinical expression of the syndrome.

More recent analyses revealed effects of additional genetic loci, in particular the human leukocyte antigen (HLA) complex, on certain disease manifestations of APECED [70, 185]. Associations with specific HLA haplotypes have been found for trait components like alopecia, AD, and T1D in patients with APECED. These haplotypes are those associated with the common, non-APECED-related forms of the specific disorder. However, only a weak association has been observed between the HLA type and autoantibody specificities in APECED patients, suggesting that in APECED the HLA alleles do not have a strong influence on autoantibody formation [185]. To date, there are only few studies on the functionality of peripheral immunological tolerance mechanisms in patients with APECED. An impairment of $CD4^+CD25^+$ Tregs in adult APECED patients has been reported [186]. However, the reduction in circulating Tregs might also be secondary to the chronic fungal infection in these individuals.

Recently, several genetic, environmental, and molecular factors potentially implicated in the phenotypic variability of APECED were investigated in two siblings affected with APECED. They were characterized by an extremely different phenotypic expression despite an identical mutation of *AIRE* (IVS1 + 1G>C; IVS1 + 5delG mutation) [162]. In particular, the younger sister had a mild form of the syndrome, while the older male developed a severe phenotype exhibiting an accelerated phase involving parathyroid, thyroid, oral mucosa, skin, liver, adrenal glands, bowel, and stomach, culminating in a life-threatening posterior encephalopathy syndrome never described before in the context of APECED [163]. So far, there is no evidence that the severity of the disease is influenced by sex in that, unlike other autoimmune diseases, APECED affects in equal manner males and females. The sibs were compared for exposure to infectious triggers (rubella, Epstein Barr virus, cytomegalovirus, toxoplasma, varicella zoster virus, parvovirus B19, herpes simplex virus, and parainfluenza virus) [187, 188], autoantibodies profile, mechanisms of peripheral tolerance (Fas-induced apoptosis, number of $TCD4^+CD25^+$ regulatory cells, and NK activity) and HLA haplotype. The results suggested that differences in the exposure to common triggering infectious agents or functionality of mechanisms governing peripheral tolerance were not involved in the clinical variability between the two sibs. However, there is the possibility that the difference in the genetic pattern between the two siblings could be responsible for the high variability in the clinical course. In fact, for APECED, as for other mendelian disorders, the interplay between multiple genetic, epigenetic and environmental factors certainly play a role in phenotypic variability of APECED. As APECED represents a paradigmatic example of monogenic disease with a very heterogeneous clinical expression, other complex autoimmune diseases are characterized by a strong genetic inheritance although not related to a single gene mutation.

Autoimmune thyroid disease (AITD) is an example of complex interaction between genetic, epigenetic, and environmental factors. Epidemiological data, including family and twin studies, revealed a strong genetic susceptibility with a positive family in about half of the patients. Moreover, genetic anticipation, which is common in mendelian diseases, has been documented in cohorts of AITD families [189]. Taken together, these observations suggest that the inheritance of an "at-risk" genotype creates a susceptible background predisposing to development of AITD. However, AITD is characterized by an extreme variability in the clinical expression even

when familial inheritance is well documented. To unravel the intrinsic mechanisms of the genetic influence and the modulation of clinical expression in AITD, several genes, mainly involved in the cross-talk between APCs and T cells in the immunological synapse, have been studied. Main susceptibility genes are summarized in Table 1. These genes can be divided as follows: immunological synapse genes (HLA-DR, cytotoxic T-lymphocyte antigen-4 [CTLA4], lymphoid tyrosine phosphatise non-receptor [PTPN22], and B lymphocyte surface immunoreceptor [CD40]) (Figure 3), T-regulatory gene (FOXP3, IL-2RA), and thyroid-specific genes (thyroglobulin and TSH receptor [TSHR]).

Studies on all these genes have revealed that, although several polymorphic variations of all the cited genes have been identified and linked to AITD, most of them have only a weak effect on the increased genetic susceptibility to develop the disease. Familial clustering in complex autoimmune diseases does not necessarily mean that the disease is genetic in nature. Familial clustering can also be stochastic, or result from a combination of shared extrinsic and intrinsic factors. On the other hand, it is possible that in monogenic disease, an interplay between multiple genetic and molecular factors may be involved in modulating the clinical expression of the disease.

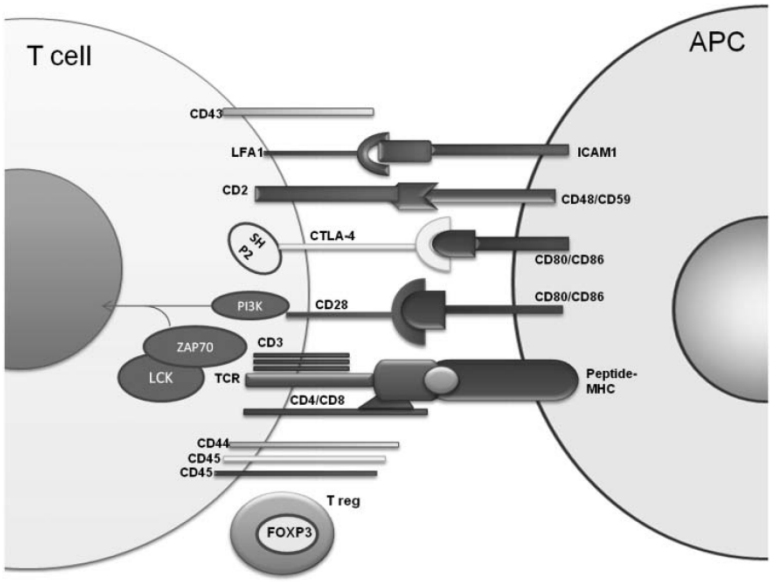

FIGURE 3. Immunological synapse. A summary view of the key ligand pairs and signaling molecules that are involved in T-cell recognition. CD80/CD86 is stimulatory peptide-MHC molecule; CD48/CD59, ICAM1, LFA1, CD2, CD28, CD3, CD4/CD8, and CD45 are activating/co-stimulatory molecules; CTLA-4 is an inhibitory molecule; and CD43 and CD44 do not contribute to signaling. The arrow indicates converging signals that lead to T-cell activation. APC, antigen-presenting cell; CD44/45, cell-surface glycoproteins; CTLA4, cytotoxic T-lymphocyte antigen 4; FOXP3, forkhead box P3; ICAM1, intercellular adhesion molecule 1; LFA1, leukocyte function-associated antigen 1; PI3K, phosphatidylinositol 3-kinase; SHP2, SRC homology 2-domain-containing protein tyrosine phosphatase 2; TCR, T-cell receptor; T reg, regulatory T-cell; ZAP70, ξ-chain-associated protein 70.

TABLE 1. Susceptibility genes for autoimmune thyroid disease (AIDT)

Candidate gene	Chromosome	Function
Human leukocyte antigen (HLA)-DR	6p21	Located on professional antigen-presenting cells (APCs) and involved in presenting peptides to lymphocytes
Cytotoxic T-lymphocyte associated protein 4 (CTLA4)	2q33	Negative regulation of T-lymphocyte activation
Protein tyrosine phosphatase non receptor 22 (PTPN22)	1p13	Lymphoid-specific intracellular phosphatase involved in regulating the T-cell receptor signaling pathways
B-lymphocyte surface immunoreceptor (CD 40)	20q	Expressed primarily on B cells and other APCs; important role in B-cell activation and antibody secretion
T-regulatory gene (FOXP3)	Xp11.23	Key gene for the differentiation of T cells into natural Treg cells
Interleukin 2 receptor α-chain (IL2RA)	10p15	Element of the high-affinity IL2 receptor, involved in IL2 signaling; present on many T-cell subsets; regulator of Treg cells
Thyroid-stimulating hormone receptor (TSHR)	14q31	G-protein-coupled receptor with a central role in controlling thyroid cell metabolism
Thyroglobulin (Tg)	8q24	Involved in the production of the thyroid hormones thyroxine (T4) and triiodothyronine (T3)

CONCLUSIONS

Recent evidence indicates that systemic autoimmunity and immunodeficiency can be strictly linked. The discovery of genetic diseases caused by alterations of genes implicated in the tolerance mechanisms enormously contributed to our understanding of the molecular basis of human autoimmune disorders, generally and appropriately considered as multifactorial diseases. The paradigm of the genetically determined alteration of central tolerance is related to mutations of the AIRE transcription factor. This disease gave a huge amount of information on the functional and molecular events that lead to the elimination of self-reactive T-cell clones within the thymus. The elucidation that TSA are broadly expressed by mTECs also helped in understanding the mechanism by which autoimmunity may also paradoxically be associated with an immunodeficiency status. However, the great variability of APECED phenotype implies the participation of several disease-modifying genes and environmental factors to the disease phenotypic expression. Since these elements are presumably pathogenetically relevant in inducing nonmonogenic autoimmune disorders, such as AITD, and to explain the autoimmune susceptible background, which is very impressive in certain families, studies of these monogenic models of disease may help unravel the pathogenesis of autoimmunity. In the near future, total exome sequencing could be a good perspective from which to analyze genetic variations involved in inheritance and clinical expression of autoimmune diseases.

Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Review

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EXPERT REVIEWS

Alterations of the autoimmune regulator transcription factor and failure of central tolerance: APECED as a model

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Vera Gallo[1], Giuliana Giardino[1], Donatella Capalbo[1], Loredana Palamaro[1], Rosa Romano[1], Francesca Santamaria[1], Filomena Maio[1], Mariacarolina Salerno[1], Pietro Vajro[2] and Claudio Pignata*[1]

[1]*Department of Pediatrics, 'Federico II' University, S Pansini 5, 8013 Naples, Italy*
[2]*Department of Medicine and Surgery, Chair of Pediatrics, University of Salerno, Italy*
**Author for correspondence:*
Tel.: +39 081 746 4340
Fax: +39 081 545 1278
pignata@unina.it

Self–nonself discrimination plays a key role in inducing a productive immunity and in preventing autoimmune reactions. Central tolerance within the thymus and peripheral tolerance in peripheral lymphoid organs lead to immunologic nonresponsiveness against self-components. The central tolerance represents the mechanism by which T cells binding with high avidity to self-antigens are eliminated through the so-called negative selection. Thymic medullary epithelial cells and medullary dendritic cells play a key role in this process, through the expression of a large number of tissue-specific self-antigens involving the transcription factor autoimmune regulator (AIRE). Mutations of *AIRE* result in autoimmune polyendocrinopathy candidiasis ectodermal dystrophy, a rare autosomal recessive disease (OMIM 240300), which is the paradigm of a genetically determined failure of central tolerance and autoimmunity. This review focuses on recent advances in the molecular mechanisms of central tolerance, their alterations and clinical implication.

KEYWORDS: AIRE • autoimmunity • autoimmune polyendocrinopathy candidiasis ectodermal dystrophy • central tolerance • phenotypic variability • susceptibility genes

The thymus represents the central organ of immunologic self–nonself discrimination. Within the thymus, developing thymocytes undergo positive and negative selection, resulting in mature T cells that are able to successfully recognize a wide range of foreign antigens and, at the same time, able to ignore self-antigens. Potentially, autoreactive naive T cells, which succeed in reaching the periphery, are functionally suppressed by several mechanisms, in a process known as peripheral tolerance. Breakdown of the central or peripheral tolerance leads to the development of destructive autoimmune reactions and, paradoxically, in a few circumstances also to immunodeficiency, as seen in experimental conditions regarding alteration of the transcription factor autoimmune regulator (AIRE) protein or FOXP3. Recent evidence suggests that systemic autoimmunity and immunodeficiency can be strictly interconnected. In immunodeficiencies, the decreased or abolished capacity of the immune system to clear infections causes a continuous immunological stimulation and activation, which represents one of the potential mechanisms that eventually leads to autoimmunity. Dysregulation of immunoregulatory factors combined to multiple genetic variations is responsible for systemic autoimmunity. In a few cases, along with a predominant autoimmune phenotype, an increased susceptibility to infections is also present [1].

Several studies have reported evidence indicating that autoimmune phenomena occur in patients affected with primary immunodeficiencies, and have focused on the molecular and cellular mechanisms underlying these conditions, in order to elucidate the link between autoimmunity and immunodeficiency. On the other hand, studies on genetic autoimmune disorders are also extremely useful for our understanding of the pathophysiology of the intimate mechanisms that lead autoreactive clones to escape clonal deletion [2,3].

Currently, unique models, which help unravel many aspects of the development, homeostasis and regulatory properties of the immune system, are the monogenic autoimmune disorders and, in particular, autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED), immunodysregulation, polyendocrinopathy and enteropathy X-linked (IPEX), autoimmune

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lymphoproliferative syndrome and IL-2 receptor α-chain (IL-2RA, CD25) deficiency.

Impairment of central or peripheral tolerance, which normally prevents the survival, expansion and activation of autoreactive T cells, thus protecting from autoimmune diseases, is the main pathogenetic mechanism responsible for these conditions. Since an important contribution to our understanding of autoimmunity was given by genetic models of abnormal central tolerance, in this review, the authors focus on APECED pathogenesis, a paradigmatic example of a disease due to the failure of central tolerance.

Functional & molecular mechanisms governing the central tolerance

Positive & negative selection

Tolerance represents a state of immunologic nonresponsiveness in the presence of a particular antigen. The immune system is able to discriminate between self- and nonself-antigens. This property is fundamental to induce a productive immunity and to prevent autoimmune reactions. Altering this balance will result in immunodeficiency or autoimmunity. Immune tolerance to self-antigens is acquired through two main processes: central and peripheral tolerance [4]. Central tolerance takes place within the thymus through the negative selection process, which occurs subsequently to the positive one. Eventually, the tolerance to self-antigens expressed in various tissues is acquired [4].

T-cell precursors originate in the bone marrow, like B lymphocytes, but, unlike these cells, complete their development within the thymus. After entering the thymus, immature lymphocytes make a commitment to the αβ T-cell lineage, rearranging first the T-cell receptor (TCR)β chain and then the TCRα chain in a sequential manner. At this stage, developing double-positive (DP) thymocytes, expressing both CD4 and CD8 on the cell surface, with functional TCR can be positively selected by peptide–MHC in the thymic cortex. The majority of DP thymocytes do not receive a rescue signal through TCR and, therefore, undergo 'death by neglect' or apoptosis. Only approximately 5% of DP cells are able to bind an MHC ligand with mild avidity, inducing their maturation to the $CD4^+CD8^-$ or $CD4^-CD8^+$ single-positive (SP) cells (Figure 1) [5]. Positively selected DP cells trigger the expression of chemokines that direct the T cells through the subsequent maturation step within the thymic medulla [6]. Here, the last stage of T-cell development takes place, the so-called negative selection, a fundamental process to establish central tolerance. This process enables T cells binding self-antigens with high avidity, potentially autoreactive lymphocytes, to be eliminated (Figure 1) [4,7]. Subsequently, thanks to the mechanisms of peripheral tolerance, autoreactive lymphocytes that have escaped negative selection are deleted in the periphery [4].

Within the thymic medulla, thymic medullary epithelial cells (mTECs) and medullary dendritic cells (mDCs) play a central role in the establishment of self-tolerance through the negative selection process [8]. Both cellular types, expressing the costimulatory molecules, CD40, CD80, CD86 and MHC class II, are able to favor an efficient thymocyte deletion. mTECs express a thousand genes, the so-called 'promiscuous gene expression', including tissue-specific self-antigens (TSAs), normally present only in specialized peripheral organs [9]. This high expression by mTECs of genes encoding TSAs is driven by AIRE [10–12]. TSAs include, among other tissues, proteins restricted to the pancreas, stomach, eye, salivary gland, muscle and thyroid [10,13,14]. These MHC-restricted self-antigens are presented by mTECs to developing T cells. Thymocytes that recognize self-antigens with high affinity/avidity are deleted [15]. Remarkably, not all TSAs seem to be regulated by AIRE, since the expression of some of them, such as acid decarboxylase (GAD67), is not altered in AIRE deficiency. In fact, AIRE controls TSAs' gene expression to variable degrees, some genes being strongly dependent on its activity, while others weakly or not at all dependent, such as C-reactive protein and GAD67. Although no definitive explanation for this phenomenon is available, certainly, further genetic and/or epigenetic mechanisms might be involved in the regulation of the complex promiscuous gene-expression process in mTECs. Unfortunately, to date, they still remain unraveled, in spite of the increasing knowledge of the AIRE mechanism of action. It is surprising that a low number of mTECs are able to delete such a huge number of clonotypes, since only a few mTECs express a given TSA [11,12]. A possible explanation is that during a 4- to 5-day period, there is a high motility of thymocytes, allowing a huge number of interactions with mTECs [16]. mDCs have a similar role, although the expression of self-antigens on the cell surface is mostly dependent on the phagocytosis of apoptotic mTECs [17,18].

Some of the thymocytes, which bind self-MHC–peptide complexes with high affinity, express *Foxp3*, and through a yet unknown mechanism escape negative selection and differentiate into Tregs, which play an important role in the peripheral tolerance suppressing autoreactive T cells in the periphery [19,20].

Triggering the costimulatory molecules and the subsequent activation of Fas signaling pathway represents the commonly accepted mechanism to explain the deletion of self-reactive thymocytes within the thymus [21]. Indeed, the costimulatory molecules expressed by mTECs and mDCs, such as CD40, CD80 and CD86, are involved in the process [22]. Moreover, B7 molecules, which interact with thymocyte CD28 receptor and are expressed by antigen presenting cells, also represent a crucial costimulatory factor [23]. Indeed, studies have shown that exposure of DP thymocytes to anti-TCR monoclonal antibody (mAb) fails to induce thymocyte death, but not in the presence of antigen presenting cells or in the combined presence of anti-TCR and anti-CD28 mAb *in vitro* [23]. Furthermore, it has been observed in SP thymocyte cultures that the exposure to high concentrations of anti-TCR mAbs is able to induce apoptosis through high-level TCR signaling in the absence of any costimulation [23]. Thus, in this condition, the strong TCR ligation leads to apoptosis in a Fas-dependent manner [24]. This effect is abolished in Fas-lpr/lpr cells [24], carrying homozygous mutation of Fas. Moderate-avidity TCR binding with costimulatory molecules induces cell death in a Fas-independent manner.

AIRE

In humans, the *AIRE* gene maps to chromosome 21q22.3 and has been cloned simultaneously in 1997 by two independent research groups [25,26]. It consists of 14 exons spanning 11.9 kb of genomic DNA [27] and encodes a 545 amino-acid protein with a molecular weight of approximately 58 kDa [25].

The AIRE protein is mainly localized in the cell nucleus, within nuclear bodies distributed near to nuclear speckles [28], suggesting the involvement of AIRE in the regulation of gene transcription. This hypothesis has also been confirmed by structural characteristics of the protein. Three different isoforms of AIRE protein, generated by alternative splicing, have been identified. These different isoforms show a unique combination of domains, common to transcriptional regulators and chromatin-binding proteins. The N-terminal region shows a six-helix structure with high similarity to a caspase-recruitment domain [29], which is necessary for AIRE oligomerization and localization to promyelocytic leukemia nuclear domains [30]. Moreover, AIRE contains a Sp100, AIRE1, NucP41/75, DEAF1 (SAND) domain [31] important for AIRE transactivation capacity and subcellular localization [32]. The C-terminal region contains two plant homeodomain PHD-type zinc fingers [33], which, if mutated induce a severe decrease of AIRE transcriptional activation capacity [34]. Other structural features include a conserved nuclear targeting signal, four LXXLL motifs or nuclear receptor interaction domains, which are found on coactivators of nuclear receptors and proline-rich regions and are also associated to transcription regulation [35].

In order to study the function of AIRE, transgenic mouse models were generated. The murine *Aire* gene has been mapped to chromosome 10. It shows a strict similarity of the genetic sequence and structural organization with the human ortholog [36]. Studies of Aire knockout mice (*Aire*$^{-/-}$) have proved to be an important tool in furthering our understanding of how *Aire* prevents autoimmunity. The gene-expression profile of mTECs from *Aire*$^{-/-}$ has shown a reduced expression of many TSAs, compared with wild-type mice. Accordingly, *Aire*$^{-/-}$ mice show several autoimmune manifestations, lymphocytic infiltrates in multiple organs and autoantibodies against several peripheral organs and tissues, including the salivary gland, retina, pancreas, stomach, ovary and thyroid [37]. However, *Aire*$^{-/-}$ mice do not exhibit increased susceptibility to *Candida* infections [38].

Nevertheless, the precise molecular mechanisms that AIRE uses to regulate transcription remain to be clarified. AIRE acts as a coactivator in a large transcriptional complex, since the vast majority of *AIRE*-regulated genes are arranged in chromosomal clusters [39]. Moreover, recent studies have demonstrated that AIRE binds a large array of partners (Figure 2) [40]. AIRE's partners could be divided into four major functional classes: nuclear transport, chromatin binding/structure, transcription and pre-mRNA processing factors [40]. The first protein reported to bind to AIRE was CREB-binding protein [41]. CREB-binding protein acetylates both histone and nonhistone proteins and is important for the initiation of transcription. After its interactions with AIRE, histone acetylation and recruitment of chromatin transcription factors may promote gene-transcription activation [42,43]. AIRE's other partners have been identified, such as DNA protein kinase (DNA-PK) and SP-RING domain protein inhibitor of activated STAT1 (PIAS1) [44,45]. DNA-PK is a serine/threonine kinase activated by dsDNA breaks, which plays a key role in VDJ recombination [46]. DNA-PK deficiency in the stromal compartment leads to decreased TSA expression in mTECs, thus resulting in autoantibody production [44]. AIRE also binds and recruits the positive transcription elongation factor b (P-TEFb) complex to RNA polymerase II. It represents a key element enhancing the transcription–elongation process [47]. It is plausible that AIRE recruitment of all its cotranscriptional partners to TSA gene promoters, which are otherwise silenced, activates the expression of the large number of TSA in mTECs [48].

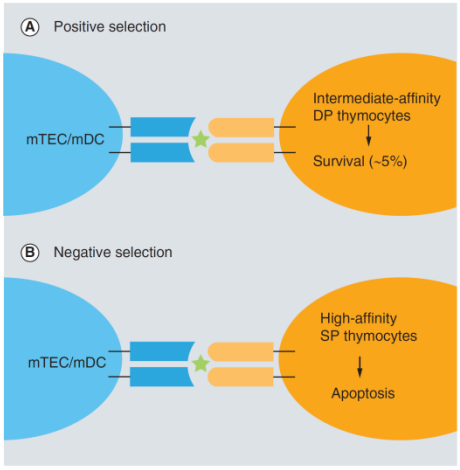

Figure 1. Positive and negative selection of developing thymocytes. (A) Positive selection of DP thymocytes, which bind with intermediate-affinity self-antigens presented by mTECs and mDCs. **(B)** Negative selection of SP thymocytes binding with high-affinity self-antigens.
DP: Double positive; mDC: Medullary dendritic cell; mTEC: Thymic medullary epithelial cell; SP: Single positive.

Accumulating evidence suggests that Aire may also play a role in the proper differentiation of the thymic medullary epithelium. Aire has been shown to be able to induce apoptosis in end-stage terminally differentiated mTECs (K5–K8^{+}) [49]. Stellate K5^{+} mTECs are normally eliminated by Aire's proapoptotic activity before completion of their terminal differentiation, and only an absence of Aire would reveal the full program of mTEC terminal differentiation ending with the globular K5–K8^{+}. Aire may also promote mTECs' differentiation program. Evidences suggest that lack of Aire in mTECs results in an arrest of the differentiation program, with the cells remaining at the premature stage just before terminal differentiation. The *Aire*$^{-/-}$ mTECs lack the transcriptional activity for Aire-dependent TSA genes [50].

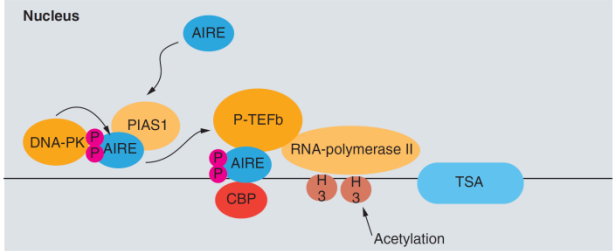

Figure 2. Autoimmune regulator's partners in the transcription regulation of tissue-specific self-antigens.
TSA: Tissue-specific self-antigen.

The *AIRE* gene is also expressed in peripheral lymphoid tissues, such as lymph nodes and spleen, at a lower degree than in thymic stromal cells. Recent studies have identified a population of extrathymic *Aire*-expressing cells in lymph nodes, corresponding to a subset of activated dendritic cells, which express TSAs and tolerogenic molecules. Presumably, also in the periphery, AIRE contributes to the process of immune tolerance by inducing TSAs' gene expression [15,51].

Alterations of AIRE expression have also been detected in patients with immunodeficiency and autoimmunity [46]. A severe reduction of AIRE mRNA and protein has been found in the thymus of two Omenn syndrome patients and one patient with T–B–NK$^+$ SCID. In addition, there was no detectable mRNA for the self-antigens insulin, CYP450 1A2, or fatty acid-binding protein in the immunodeficient patients. These findings led the authors to argue that deficiency of AIRE expression occurs in severe immunodeficiencies, characterized by abnormal T-cell development, and suggested that in Omenn syndrome, the few residual T-cell clones may have escaped negative selection and, thereafter, may eventually have expanded in the periphery, thus causing massive autoimmune reactions [52].

Central tolerance failure: the APECED model

APECED represents the prototypic disease of immune dysregulation, classified as a type IV immunodeficiency [53]. It is a rare autosomal recessive disease (OMIM 240300) caused by mutations in the *AIRE* gene [25,26] with a complex clinical phenotype discovered over decades.

Although rare, APECED has been reported worldwide [54] with a wide variability in its incidence. Epidemiologic data show a high prevalence in certain genetically isolated populations, such as Iranian Jews (1:9000) [55], Finns (1:25,000) [56] and Sardinians (1:14,400) [57]. It is also quite frequent in Norway [58] and Italy [59]. So far, more than 60 different types of mutations in the *AIRE* gene have been reported in APECED patients [14]. Different mutations have been found to be peculiar to specific areas. In Italy, an increased APECED prevalence has been found in various regions, in particular in Sardinia, Apulia and in the Venetian area. Furthermore, in both Sardinia and Apulia, two peculiar mutations of *AIRE* have been identified: the mutation R139X on exon 3 in Sardinia [60] and the W78R mutation on exon 2 in Apulia [61]. In the Veneto region, differing from the other Italian regions, *AIRE* mutations (R257X on exon 6 and 8) were similar to that identified in Finnish and Anglo-Saxon patients [62]. Recently, a typical mutation has also been identified in Sicily (R203X on exon 5) [63]. The patients from Campania show a high frequency of mutations in the exon/intron 1 junction [59].

APECED is characterized by destructive autoimmune reactions against endocrine and nonendocrine tissues and, frequently, ectodermal tissues. However, a high variable clinical expression has been well documented. Parathyroid glands, adrenal cortex, gonads, pancreatic β cells, gastric parietal cells and thyroid glands are mainly involved in the pathologic process. A few ectodermal manifestations in APECED are dental enamel hypoplasia, pitted nail dystrophy and alopecia. Furthermore, the gastrointestinal system may also be involved, leading to autoimmune gastritis, malabsorption and autoimmune hepatitis [54]. The clinical diagnosis requires the presence of at least two of the three most common clinical features, the classical triad: chronic mucocutaneous candidiasis (CMC), chronic hypoparathyroidism (CH) and Addison's disease (AD) [64]. However, if a sibling has already been diagnosed, the presence of even one component is sufficient for the diagnosis [65]. Molecular analysis of the *AIRE* gene is necessary to confirm the diagnosis and, in addition, can be helpful in those cases with atypical clinical presentation. Another specific and sensitive diagnostic tool for APECED, recently discovered, is represented by autoantibodies (autoAbs) neutralizing cytokines, in particular, type I interferons (IFN-ω and IFN-α) and Th17-related cytokines (IL-17A, IL-17F and IL-22), which emerge in an early phase of the disease, even before any clinical manifestation [66]. Organ-specific autoAbs are not used in the diagnostic procedure because of their later appearance, generally at the meantime of the corresponding autoimmune manifestation. Moreover, they are detected in 8–66% of APECED patients and are mostly not APECED-specific, but specific to the individual disease [67]. In most patients, CMC precedes the other immune disorders appearing by the age of 5 years, usually followed by CH and later by AD [68]. While a clear autoimmune pathogenesis underlies the endocrine disorders pathology, which results from destruction of the target organ by the cellular and antibody-mediated attack [65], the molecular basis of CMC and of the increased susceptibility to infections in APECED patients is still poorly understood. Indeed, autoAbs to parathyroid, adrenal glands and type I interferons are hallmarks of APECED [34,69]. Therefore, the high prevalence and early appearance of anticytokine autoAbs is very suggestive of a role in the pathogenesis of immunodeficiency. Recent studies have described a role of the autoAbs against IL-22, IL-17A and IL-17F cytokines in the pathogenesis of CMC [70,71]. CMC is a clinical feature shared by distinct

genetic diseases affecting the differentiation or functionality of Th17 lymphocytes, such as hyper-IgE syndrome. Remarkably, autoAbs against Th17-related cytokines have also been found in rare thymoma patients presenting with CMC. Of note, in the vast majority of thymomas, the neoplastic mTECs fail to express AIRE [72]. In APECED patients, IL-17F and IL-22 secretion, in particular, seems to be significantly reduced in response to *Candida albicans* [70]. All these findings allow us to speculate that CMC in APECED is also essentially autoimmune in nature and related to the development of an autoimmune response towards the IL-17 and IL-22 producing cells [73].

The current model of disease pathogenesis of APECED is based on the transgenic mouse models that showed impaired negative selection of autoreactive thymocytes in an Aire-deficient thymus. Aire deficiency in mTECs impaired the expression of a wide array of self-tissue specific antigens, which are not presented to differentiating T lymphocytes. As a consequence, potentially autoreactive T cells do not undergo negative selection and are exported to the periphery. Here, the naive autoreactive lymphocytes must also escape peripheral tolerance mechanisms to become dangerous.

Recent studies have highlighted some discrepancies in this pathogenetic model. In fact, it cannot explain the reason by which the organs affected are limited mainly to endocrine and ectodermal tissues, while AIRE controls the expression of thousands of peripheral antigens in the thymus. In addition, it should be noted that the first autoAbs to emerge in APECED patients are those against cytokines and not autoAbs implicated in organ-specific autoimmunity. Notably, cytokine antigens are not expressed by mTECs and are, therefore, not AIRE dependent [66]. According to the current model, breaking of peripheral tolerance mechanisms is also required to activate naive and autoreactive T cells reaching the periphery. All these steps need a period of time, which contrasts with the early onset of CMC, CH and AD, also taking into account the delay between initial autoimmunization and clinical manifestation, which appear only after almost a total destruction of the parathyroids or adrenal cortex occurs [74]. To explain these discrepancies, the authors have hypothesized a novel pathogentic model of 'active intrathymic autoimmunization', which alludes at additional roles for AIRE beyond regulating TSA expression [73]. According to this model, AIRE deficiency impairs the thymic microenvironment, leading to the formation of tertiary lymphoid tissue. Thymus thus becomes a site of immunization where cytokines are presented in an immunologic manner to T and B cells. Neutralizing autoantibodies against cytokines and activated autoreactive T-cells are then released directly from the thymus. After thymic export, they are already able to attack their targets [73]. Moreover, activated autoreactive T-cells against parathyroids and adrenal glands could be generated early because of the presence of parathyroid and adrenal antigens in the normal thymus. Indeed, parathyroids and thymus share the same embryological origins and it is not rare to find ectopic parathyroid tissue within the thymus. Concerning adrenal auto-antigens, several studies have reported the presence of paracrine glucocorticoid activity in the thymus. These auto-antigens should be presented by mDCs in the tertiary lymphoid infiltrates. To date, cell types and mechanisms responsible for this process remain unraveled. The two pathogenetic models are not mutually exclusive, but can coexist, the novel model explaining the earlier and most prevalent manifestations, such as autoAb responses, CMC, CH and AD (and probably gonadal failure too), and the current one explaining the later clinical features, such as diabetes, thyroid disease among others. Nevertheless, further studies are necessary to validate both the current and the emerging models of disease pathogenesis in APECED.

Phenotypic variability & genetic susceptibility

Patients affected with APECED usually show a variable clinical phenotype, in spite of the fact that APECED is a monogenic disorder. Many authors have confirmed this phenotypic and course heterogeneity among several populations, first documented in the largest reported series of 91 Finnish patients [54,56,57,59]. A precise genotype–phenotype correlation is still lacking. Notably, the clinical variability is both interfamilial and intrafamilial, the clinical features varying not only among patients from different families but also among siblings carrying the same mutation [59]. Different genetic and nongenetic factors, including modifier genes, chance and environmental interactions, as well as immune system dynamics, have a key role in modulating the clinical expression of the syndrome, even in the same family, thus explaining the intrafamilial and interfamilial variability.

Additional genetic loci, in particular the *HLA*s, have been shown to be related to certain phenotypic manifestations of APECED [14,75]. In particular, specific *HLA* haplotypes have been found to be associated to alopecia, AD and autoimmune thyroiditis in APECED patients. However, the *HLA* alleles do not have a strong influence on autoantibody formation, since only a weak association has been observed between the HLA type and the autoantibody specificities in APECED patients [75]. Several peripheral mechanisms of tolerance are also involved in the control and regulation of immune response, thus influencing the clinical expression of the disease. Natural killer cell activity represents an additional mechanism of peripheral tolerance involved in controlling the reactivity to self-antigens in the periphery. To date, because of the few studies, not much is known on the functionality of these immunological tolerance mechanisms in patients with APECED. Recently, a defect of $CD4^+CD25^+$ Tregs in APECED patients has also been reported [76]. It should be pointed out that a reduction of Tregs in APECED patients could also be observed as a secondary effect of chronic fungal infection in these individuals. On the contrary, in a recent study, an imbalance of the IL-7–IL-7R pathway leading to a loss of $CD8^+$ T-cells homeostasis has been documented in APECED patients. The abnormalities do not seem to be related to the inflammation itself. The decrease of CD127 and CD5 surface expression, along with increased levels of perforin in CD8RA cells, seems to be crucial in the breakdown of the tolerance in APECED patients [77].

Recently, two siblings affected with APECED, carrying the same mutation of *AIRE* (IVS1 + 1G>C; IVS1 + 5delG mutation), were investigated in order to detect genetic, environmental

and molecular factors potentially responsible of the intrafamilial phenotypic variability of APECED. Indeed, they were characterized by an extremely different phenotypic expression despite the identical mutation [59]. The younger sister, in particular, had a mild form of the syndrome, while the older male developed a severe phenotype exhibiting an accelerated phase involving parathyroid, thyroid, oral mucosa, skin, liver, adrenal glands, bowel and stomach, culminating in a life-threatening posterior encephalopathy syndrome never described before in the context of APECED [78]. APECED, different from the other autoimmune diseases, shows the same incidence between males and females and, so far, there is no evidence that the severity of the disease is influenced by sex. Exposure to infectious triggers (rubella, Epstein–Barr virus, cytomegalovirus, toxoplasma, varicella zoster virus, parvovirus B19, herpes simplex virus and parainfluenza virus) [79], autoantibody profile, mechanisms of peripheral tolerance (Fas-induced apoptosis, number of $TCD4^{+}CD25^{+}$ regulatory cells and natural killer activity) and HLA haplotype were compared in the two siblings. The clinical variability observed between the two siblings is not related to the differences in the exposure to infectious agents or functionality of mechanisms governing peripheral tolerance. This evidence suggests that the phenotypic variability of APECED may derive from the interaction between multiple genetic, epigenetic and environmental factors.

Expert commentary & five-year view

Recent evidence indicates that systemic autoimmunity and immunodeficiency can be strictly linked. Molecular mechanisms involved in central tolerance, along with those in peripheral tolerance, play a crucial role in the establishment and maintenance of immune self-tolerance, preventing autoimmunity and promoting the proper function of immune system. The study of genetic diseases caused by alterations of genes implicated in the tolerance mechanisms give an enormous contribution to the elucidation of the molecular basis of human autoimmune disorders, generally considered as multifactorial diseases. APECED represents a paradigmatic example of monogenic disease due to mutations of the autoimmune regulator AIRE, which leads to central tolerance failure. This disease has been extremely useful for our understanding of the functional and molecular events that lead to the elimination of self-reactive T-cell clones within the thymus. Experimental evidence indicates that AIRE acts as a transcription factor, which upregulates the expression of TSA in mTECs allowing the negative selection of potentially autoreactive T-cells. The recent discovery of autoAbs directed towards Th17-related cytokines suggested an additional role for AIRE, which leads to argue a parallel pathogenetic hypothesis for APECED that also explains CMC pathogenesis, representing a puzzle to date. Moreover, evidence that IL-22 and IL-17F are crucial in protection against CMC infection has important therapeutic implications. The elucidation that TSAs are broadly expressed by mTECs also helped understand the mechanism by which autoimmunity may also be paradoxically associated with an immunodeficiency status. However, much more remains to be learned about the role of AIREs and its precise mechanism of action. Further studies are needed to improve our understanding of the molecular mechanisms of central tolerance involving the AIRE protein. This future research is not only important for the basic understanding, but may also have important clinical and therapeutic implications. Indeed, additional proteins or pathways may be identified, which could be used as diagnostic tools or as a target of novel therapeutic interventions to prevent autoimmunity.

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Key issues

- Immune self-tolerance represents a state of immunologic nonresponsiveness against the organism's own components. It is a fundamental property of the immune system, which prevents autoimmunity.
- Central tolerance mechanisms are the processes through which immune self-tolerance is established within the thymus.
- Negative selection is considered the main mechanism involved in central tolerance, by which potentially autoreactive T-cells bind self-antigens with high avidity and are eliminated.
- Autoimmune regulator protein acts as a transcription factor, which regulates the promiscuous thymic expression of tissue-specific self-antigens by thymic medullary epithelial cells and antigen presenting cells.
- Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) is the result of mutations in the *AIRE* gene and represents the prototypic monogenic disease due to a failure of the central tolerance mechanisms.
- The pathogenetic mechanisms of APECED are, however, not completely understood. Recent evidence has shown a presumptive role of autoantibodies anti-Th17-related cytokines in APECED pathogenesis.
- The novel theory leads us to hypothesize a new pathogenesis based on an 'active intrathymic autoimmunization', which alludes at additional roles for autoimmune regulator protein.
- Further studies are required to define in detail APECED pathogenesis, which could have remarkable clinical relevance, both in diagnostics and therapeutics.

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CASE REPORT Open Access

Acute adrenal failure as the presenting feature of primary antiphospholipid syndrome in a child

Nicola Improda, Maria Alessio, Donatella Capalbo, Giustina Russo, Ida D'Acunzo, Loredana Palamaro, Claudio Pignata and Mariacarolina Salerno*

Abstract

Introduction: Antiphospholipid syndrome (APS) is characterized by recurrent arterial and venous thrombosis and detection of antiphospholipid antibodies (aPLs). This syndrome may be associated with connective tissue disorders, or with malignancies, but it may also appear in isolated form (primary APS). We report on a pediatric patient presenting with acute adrenal failure as the first manifestation of primary APS.

Case report: A previously healthy 11-year-old boy developed fever, abdominal pain, and vomiting. An abdominal computed tomography scan showed nodular lesions in the adrenal glands. He was referred to our Department and a diagnosis of APS and acute adrenal failure was considered, based on positive aPLs (IgG and IgM), elevated ACTH levels and low cortisol levels. Other features were anemia, thrombocytopenia, elevated inflammatory parameters, hypergammaglobulinemia, prolonged partial thromboplastin time, positive antinuclear, anticardiolipin, anti-platelet antibodies, with negative double-stranded DNA antibodies. Lupus anticoagulant and Coomb's tests were positive. MRI revealed a bilateral adrenal hemorrhage. A treatment with intravenous metylprednisolone, followed by oral prednisone and anticoagulant, was started, resulting in a progressive improvement. After 2 months he also showed hyponatremia and elevated renine levels, indicating a mineralcocorticoid deficiency, requiring fludrocortisones therapy.

Conclusion: The development of acute adrenal failure from bilateral adrenal haemorrhage in the context of APS is a rare but life-threatening event that should be promptly recognized and treated. Moreover, this case emphasizes the importance of the assessment of aPLs in patients with acute adrenal failure in the context of an autoreaction.

Keywords: Adrenal insufficiency, Adrenal hemorrhage, Antiphospholipid syndrome, Thrombotic events

Background

Primary adrenal insufficiency is a life-threatening event, resulting from destruction or dysfunction of the adrenal cortex. Signs and symptoms of adrenal insufficiency appear when more than 90% of the cortex is destroyed [1]. It is a rare disease with a prevalence in developed countries of 90–140 per million, but it is thought to be even less common in childhood [2]. Signs and symptoms may be nonspecific and therefore the diagnosis is often delayed, with risk of severe morbidity or mortality [3]. Recent data suggest that the most common causes of acquired primary adrenal insufficiency are autoimmune adrenalitis and tuberculosis, whereas only 5% of the cases are related to unusual disorders [4].

Adrenal insufficiency has been described in APS and is thought to be the result of hemorrhage of adrenal glands. APS is an autoimmune disorder characterized by the persistent detection of antiphospholipid antibodies (aPLs) and various clinical manifestations, the most common being venous and arterial thrombotic events, recurrent fetal loss, thrombocytopenia, livedo reticularis and neurological manifestations. APS may occur in the context of another autoimmune disease (secondary APS), or may be a primary APS. In rare circumstances, APS is defined as catastrophic due to an accelerated form of multiorgan failure for an uncontrolled thrombosis [5].

Addison's disease is reported in 0.4% of patients with ascertained APS [6]. On the contrary, APS is diagnosed in less than 0.5% of all patients with Addison's disease [7]. To date, there are only a few reports in the literature

* Correspondence: salerno@unina.it
Department of Pediatrics, Federico II University, Naples, Italy

of association between primary adrenal insufficiency and primary APS in pediatric patients.

Here we report on a 11-year-old boy with acute adrenal failure, due to bilateral adrenal hemorrhage, as the presenting feature of a primary APS.

Case report

A previously healthy 11-year-old boy developed persistent fever, abdominal pain and vomiting. An abdominal ultrasound echography was normal. An abdominal computed tomography scan showed nodular lesions in the adrenal glands. He was referred to our Department, where the diagnosis of acute adrenal failure was made on the basis of the clinical phenotype, mild hyponatremia (132 mmol/l), high plasma ACTH level (961 pg/ml n.v. 10–130), low plasma cortisol levels (31,5 ng/ml n.v. 50–200), and normal aldosterone and renine levels. MRI revealed a bilateral adrenal hemorrhage. Routine analysis revealed anemia, thrombocytopenia, elevated inflammatory parameters, hypergammaglobulinemia and prolonged partial thromboplastin time. A subclinical hypothyroidism (SH) was diagnosed on the basis of mild increase of TSH with normal levels of FT4 [8,9], in the absence of thyroid autoantibodies. Subsequent diagnostic work-up showed positive antinuclear, antiphospholipid (IgG 20,9 IgM 27,3 n.v. <10), anticardiolipin antibodies (56,6 U/ml n.v. 0–20), anti-platelet autoantibodies and negative double-stranded DNA antibodies. Lupus anticoagulant and Coomb's test were positive. A diagnosis of antiphospholipid syndrome was performed.

Main causes of primary adrenal insufficiency were ruled out. In particular, autoimmune Addison, a frequent cause of acquired adrenal insufficiency also in the context of APECED or others autoimmune syndromes [10-13], was ruled out by clinical and biochemical evaluation [14]. Family history was negative for APS or any other autoimmune diseases.

A treatment with intravenous metylprednisolone, followed by oral prednisone and anticoagulant, was promptly started, resulting in a progressive improvement. TSH levels spontaneously improved not requiring L-Thyroxine therapy [15,16]. Two months later, mineralcorticoid deficiency was diagnosed on the basis of hyponatremia, low aldosterone and elevated renine levels (>300 pg/ml), requiring fludrocortisone replacement therapy. The patient is currently on cyclosporine therapy because of the recurrence of thrombocytopenia and positivity of lupus anticoagulant and Coomb's tests, and antiphospholipid antibodies. His general conditions are stable.

Discussion

Adrenal failure is a rare but potentially life-threatening event. In childhood it generally presents with nonspecific signs and symptoms, such as fatigue, malaise, abdominal pain, nausea and vomiting. Therefore, diagnosis and treatment may be often delayed [3].

Main causes of congenital and acquired primary adrenal insufficiency are reported in Table 1 [17]. Adrenal hemorrhage is a rare but well-known cause of adrenal insufficiency. In a recent retrospective study on the presentation of primary adrenal insufficiency in childhood, adrenal haemorrhage was reported in only 2 of 77 cases (3%) detected between 1999–2010 [3]. Adrenal glands are highly susceptible to hemorrhagic damages [18].

The presence of antiphospholipid antibodies is a major risk factor for hemorrhagic infarction of the adrenal glands as a consequence of a thrombotic event [19]. The detection of antiphospholipid antibodies in a subject with thrombosis should lead to suspect a diagnosis of APS. Criteria to confirm the diagnosis of APS include at least one clinical sign, such as vascular thrombosis or pregnancy complications, and one biochemical criterion such as anticardiolipin IgG or IgM antibodies, lupus anticoagulant of IgG or IgM classes detected in two occasions at least 6–12 weeks a part [7,20]. Recent evidence demonstrates that aPLs may recognize the

Table 1 Main causes of primary adrenal failure

Congenital	CAH
	Congenital adrenal hypoplasia
	ACTH resistance
	Glucocorticoid resistance
	Metabolic diseases (Adrenoleukodystrophy, Zellweger, Smith-Lemli-Opitz, Wolman disease)
Acquired	Autoimmune adrenalitis
	- Isolated
	- Autoimmune polyendocrinopathy
	- syndrome type 1
	- Autoimmune polyendocrinopathy syndrome type 2
	Hemorrhage/infarction
	- Trauma
	- Waterhouse-Frederickson syndrome
	- Anticoagulation
	- Thrombosis (APS, Thrombophilia)
	Drug effects (Aminoglutethimide, mitotane, ketoconazole, medroxyprogesterone)
	Infection
	- Viral: HIV, cytomegalovirus
	- Fungal: coccidiomycosis, histoplasmosis, blastomycosis, cryptococcosis
	- Mycobacterial: tuberculosis
	- Amebic
	Infiltrative (Hemochromatosis, histiocytosis, sarcoidosis, amyloidosis, neoplasm)

β2-glycoprotein I bound to the surface of resting endothelial cells, whose activation switches them to a procoagulant and pro-adhesive phenotype. Subsequently, the great majority of clinical signs of APS, including adrenal failure, are related to recurrent venous, arterial or small-vessel thrombosis [21,22].

Besides vascular occlusion a great variety of non-thrombotic manifestations may be associated [5,20]. Our patient had hematological complications, such as hemolytic anemia and thrombocytopenia. The clinical spectrum of antiphospholipid syndrome is very broad and primary adrenal failure is considered a rare manifestation, particularly, in childhood.

Espinosa and colleagues reviewed all cases of primary adrenal insufficiency associated with APS described in the literature from 1983 (when APS was first defined) through March 2002. Of the 86 patients identified, 6 (3 males) were less than 18 years old; 5 of these cases had a primary APS and only one patient was diagnosed with a SLE-like syndrome. Moreover, in two patients Addison's syndrome was the first manifestation of APS, while in two children adrenal insufficiency developed in the context of catastrophic APS. Furthermore, in two cases precipitating factors were identified, such as neonatal infection and pneumonia [1].

In a recent study, Presotto et al. identified, throughout a computer-assisted search of the literature from 1988 through January 2005, 19 cases of primary adrenal insufficiency as the heralding symptom of primary APS. 4 patients (2 males) were less than 18 years old and in 1 case a precipitating factor was identified [7]. Massive bilateral adrenal hemorrhage has been also described in a newborn with primary antiphospholipid syndrome hypothesized basing on the persistent detection of anticardiolipin antibodies after 6 months of life [18].

In our case, bilateral adrenal haemorrhage appeared to be the first expression of a hypercoagulable state due to the APS in the absence of any precipitating factor.

Of note, in our patient, the fact that the mineralcorticoid activity was damaged only later suggested a centrifugal progression of adrenal impairment from the zona reticularis to the zona glomerulosa, consistent with a thrombosis of the central adrenal vein(s).

Conclusion

In conclusion, the development of acute adrenal failure due to bilateral adrenal haemorrhage in the context of antiphospholipid syndrome is a rare but life-threatening event that should be promptly recognized and treated. Moreover, our case emphasizes the importance in the assessment of antiphospholipid antibodies in all patients with rapidly progressive acute adrenal failure in particular when other autoimmune signs and symptoms are present.

Consent

Written informed consent was obtained from the parents of the patient for publication of this Case report and any accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

Abbreviations

APS: Antiphospholipid syndrome; aPLs: Antiphospholipid antibodies.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contribution

All authors have equally participated in drafting of the manuscript and/or critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript.

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§2.1.2 Altered peripheral tolerance and autoimmune disease

The paradigm of abnormal peripheral tolerance is ALPS, a disorder characterized by nonmalignant lymphoproliferation, increased risk of lymphoma, and autoimmunity often manifesting as multilineage cytopenias [98,99] The most common genetic alterations are heterozygous germline mutations in the gene encoding the TNF receptor family member Fas (CD 95, Apo-1) [100-102], somatic Fas mutations and mutations in the genes encoding Fas-ligand (FASLG), caspase 10 (CASP10) and caspase 8 (CASP8), and NRAS and KRAS [103]. However, in a large group of ALPS patients the molecular defect still remains to be identified.

Alterations of homeostatic mechanism resulting in an abnormal lymphocyte accumulation, autoimmunity or lymphoid malignancies, have now emerged as a novel pathogenic mechanism underlying intense poly-reactive auto-reactions [104,105]. Recent evidence indicates that, in a few cases, Clustering of Autoimmune Disorders (CAD) may represent unique model of monogenic autoimmune disorder or a sign of congenital immunodeficiencies [106-108]. CAD has been defined by the presence of at least three distinct organ-specific or systemic immune disorders in the same individual [109]. We reported on a functional impairment of cell death, induced through Fas triggering, in the 60% of a cohort of patients affected with CAD, thus suggesting some overlap with ALPS [104,110]. Only in 1 patient the functional alteration was associated with Fas gene (TNFRSF6) mutation. Our study highlights the importance to evaluate Fas-induced cell survival in the clinical approach to patients with CAD even though the exact role of Fas-induced cell death abnormalities in the pathogenesis of CAD remains to be fully elucidated.

The results of this study were published on *Italian Journal of Pediatrics,* for the manuscript see below.

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LETTER TO THE EDITOR Open Access

Altered regulatory mechanisms governing cell survival in children affected with clustering of autoimmune disorders

Loredana Palamaro[1], Giuliana Giardino[1], Francesca Santamaria[1], Ugo Ramenghi[2], Umberto Dianzani[2] and Claudio Pignata[1*]

Abstract

Clustering of Autoimmune Diseases (CAD) is now emerging as a novel clinical entity within monogenic immune defects with a high familial occurrence. Aim of this study is to evaluate the regulatory mechanisms governing cell survival, paying a particular attention to Fas-induced apoptosis, in a cohort of 23 children affected with CAD. In 14 patients, Fas stimulation failed to induce cell apoptosis and in 1 case it was associated with Fas gene mutation. Our study highlights the importance to evaluate cell apoptosis in the group of children with CAD, which, with this regard, represents a distinct clinical entity.

Keywords: Clustering of autoimmune diseases, Fas, Apoptosis, ALPS

Dear Editor,

Even though distinct autoimmune disorders may be associated in the same individual [1,2], only rare patients exhibit a clear clustering of distinct diseases, which are indicative of a common poly-reactive autoimmune process [3]. Along with environmental factors, a genetic susceptibility represents a well established feature in the predisposition of individuals to certain autoimmune diseases, including the association with certain specific HLA and complement polymorphic variants. However, the intimate pathogenic mechanism of autoimmunity still remains to be unraveled. Alterations of homeostatic mechanism resulting in an abnormal lymphocyte accumulation, autoimmunity or lymphoid malignancies, have now emerged as a novel pathogenic mechanism underlying intense poly-reactive auto-reactions [4-6]. Recent evidence indicates that, in a few cases, Clustering of Autoimmune Disorders (CAD) may represent unique model of monogenic autoimmune disorder or a sign of congenital immunodeficiencies [7-9]. Hematologic autoimmune disorders associated with non-malignant lymph adenopathy are the prominent clinical features of the Autoimmune Lymphoproliferative Syndrome (ALPS), whose molecular characterization leads to define five distinct entities on the basis of the location of the defect in the Fas signaling cascade [3]. However, in a large group of ALPS patients the molecular defect still remains to be identified. We recently reported on a group of children affected with CAD who exhibited a high prevalence of familial cases [10].

Aim of this study is to evaluate Fas-induced apoptosis in this cohort of patients.

CAD was defined by the presence of at least three distinct organ-specific or systemic immune disorders in the same individual [10]. The predominant autoimmune diseases in these 23 patients (14 female) were rheumatoid arthritis, type 1 diabetes, autoimmune thyroiditis and celiac disease, as previously described in detail [10]. Fas-mediated lymphocyte apoptosis was evaluated on activated T-cell lines obtained by treating peripheral blood mononuclear cells (PBMC) with phytohemagglutinin (PHA) at days 0 (1 μg/mL) and 12 (0.1 μg/mL), as previously reported [2,11]. Fas function was defined defective when cell survival was higher than 78%, which was the 95th percentile of the response displayed by normal controls.

Fas-induced cell death in PHA-derived T-cell lines was abnormal in 14 of the 23 patients (60%) (Figure 1). Fas

* Correspondence: pignata@unina.it
[1]Department of Pediatrics, "Federico II" University, Pansini 5, 80131, Naples, Italy
Full list of author information is available at the end of the article

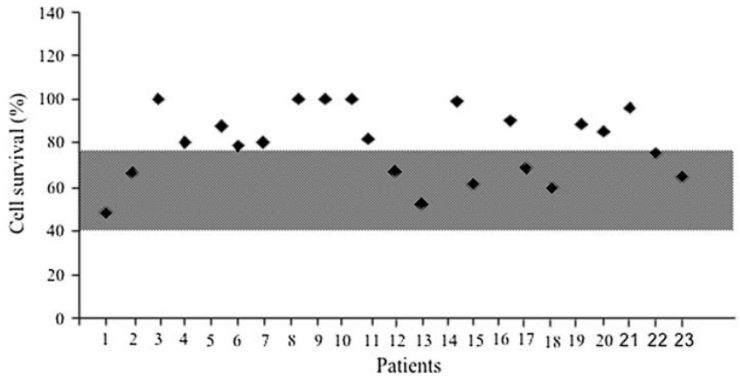

Figure 1 T-cell sensitivity to Fas induced cell death in CAD patients. Peripheral blood T cells were stimulated by PHA and cultured in the presence of rIL-2 prior to further stimulation through Fas receptor. Results were expressed as relative cell survival percentage, calculated as follows: (total live cell count in the assay wells/total live cell count in the untreated samples) x 100. Shaded area indicates the range of normal values (i.e. the range between the 5th and the 95th percentile of normal controls values).

expression was evaluated in the long-term T-cell lines by direct immunofluorescence on the same day in which Fas function was assessed and was expressed always at comparable levels than in controls. In the 14 patients with defective Fas-induced apoptosis, sequencing analysis of the Fas gene (TNFRSF6) revealed a 2 base deletion in exon 4 (g410-411delCT) in one patient (Pt#14). In other patients, 5 already described silent polymorphisms were also found, 2 of them in the 5' UTR region, 2 in the coding region, and 2 in the intronic region (IVSIII nt 46, IVSV nt 82).

In this study we report on a functional impairment of cell death, induced through Fas triggering, in the 60% of patients affected with CAD, thus suggesting some overlap with ALPS [3,4]. Only in 1 patient the functional alteration was associated with gene mutation.

A genetic cause of certain complex autoimmune syndromes has also been established in Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy syndrome (APECED) [12,13]. However, in our patients the diagnostic criteria for this syndrome were missing. Apoptosis is a complex process that plays a central mechanism in the homeostasis of immune response and in the regulation of the cellular differentiation [14,15]. It is triggered through 2 major signaling pathways [16-18]. The first involves death receptor family members, such as CD95/Fas, TRAILR1-2, TNF-R1, which in turn activate the caspases cascade, resulting in caspase 3 activation [19]. The process results in the proteolytic cleaveage of nuclear and cytoplasmic substrates, and the subsequent cellular disassembly [20,21]. Along with this Fas-dependent pathway, several stimuli, such as DNA damage, metabolic imbalance, growth factor deprivation, or cell cycle perturbation activates the alternative mitochondrial apoptotic pathway [18]. This implies that a very high number of signaling molecules involved in the processes may be altered causing an ALPS-like phenotype [22].

In conclusion, our study highlights the importance to evaluate Fas-induced cell survival in the clinical approach to patients with CAD even though the exact role of Fas-induced cell death abnormalities in the pathogenesis of CAD remains to be fully elucidated. The high prevalence of familiarity in such cases would suggest an inheritable pathogenetic mechanism, even though in previous studies it has been shown that there is no correspondence in the clinical phenotype among different family members indicating a role for several environmental and genetic factors [10,23].

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
LP participated in the study design, analysis and interpretation of data and wrote the draft of the manuscript. GG, FS have been involved in the collection of clinical data of the patients and in the interpretation of data. UR and UD carried out the molecular studies. CP designed and supervised the study. CP also wrote and approved the final version to be published. All authors read and approved the final manuscript.

Author details
[1]Department of Pediatrics, "Federico II" University, Pansini 5, 80131, Naples, Italy. [2]Interdisciplinary Research Center of Autoimmune Diseases (IRCAD) and Department of Medical Science, "A. Avogadro" University, Eastern Piedmont, Novara, Italy.

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§ 2.1.3 SCID-like phenotype associated with autoimmunity

Interestingly, evidence is emerging that, unlike total immunodeficiencies, partial T-cell immunodeficiencies are more frequently associated with hyperimmune dysregulation with a frank autoimmune phenotype [111]. Even more interesting the observation that the loss of function or gain of function alterations in the immune system functionality may arise from abnormalities of the same multiple genes, that in some cases lead to total and in other to partial deficiencies. This would imply that the hyperimmune dysregulation is not related to selected genes alterations, but rather to the partial T-cell immunodeficiency itself [111].

We reported a patient with a clinical phenotype resulting in a typical lymphocytopenic $T^-B^+NK^+$ SCID. A similar phenotype is generally due to an impairment of the T-cell differentiation process resulting in a severe reduction in peripheral T-cell pool size associated with molecular alterations of genes implicated in T-cell ontogeny and functionality [112-114].

In conclusion, this SCID-like patient was characterized by a severe T-cell activation deficiency, in whose serum an inhibitory factor, precipitated in the purified Ig fraction and able to potently inhibit control cells proliferation, was identified. So far, an autoreactive anti-lymphocyte antibody able to induce a SCID phenocopy has never been described. Notably recent studies have identified immunodeficiencies associated with the presence of autoantibody against several cytokines such as interferon (IFN)-γ , interleukin (IL)-17, IL-22.

The results of this study were published on *Journal of Investigational Allergology & Clinical Immunology*, for the manuscript see below.

In our case, the clinical and histological features were compatible with a diagnosis of Sweet's syndrome. A reaction to azathioprine was suspected due to the temporal relationship between drug administration and onset of lesions and the resolution of signs and symptoms after withdrawal. Recurrence after an oral challenge confirmed the suspected diagnosis of azathioprine-induced Sweet's syndrome. In the second episode the symptoms resolved sooner, presumably due to a shorter exposure to the drug.

The first convincing case of azathioprine-induced Sweet's syndrome was reported by Stapleton in 2003. Since then, only 6 cases with a plausible link to the use of azathioprine have been described. In 2 of these, the causal relationship was not firmly established because an oral challenge was not performed and the underlying diseases (myasthenia gravis and Crohn's disease) might have been responsible for the reactions reported. The other 4 reports were associated with inflammatory bowel disease [4-7]. In all of these cases, azathioprine was strongly implicated as the causal agent as there was a well-defined temporal relationship, resolution of lesions after drug discontinuation, and a new eruption after the reintroduction of azathioprine. In none of the cases were allergy tests carried out.

To the best of our knowledge, there are no reports of azathioprine-induced Sweet's syndrome associated with microscopic polyangiitis in the literature.

The pathogenesis of Sweet's syndrome is unknown. It has been defined as a type-III hypersensitivity phenomenon, although there is new evidence that suggests the involvement of other mechanisms, including elevated granulocyte colony-stimulating factor [8], associations with determined histocompatibility (Bw54)[9], and a possible role of antineutrophil cytoplasmic antibodies in the activation of neutrophils [10].

We emphasize the importance of evaluating azathioprine as a possible, though uncommon, cause of Sweet´s syndrome and of replacing it with a non-purine analog treatment if the involvement of azathioprine is confirmed.

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María Pilar López Sáez
Servicio de Alergología
Hospital Universitario Virgen de la Arrixaca
Carretera Cartagena, Km. 7
30120 El Palmar, Spain
E-mail: mpilarlopezsaez@yahoo.es

SCID-Like Phenotype Associated With an Inhibitory Autoreactive Immunoglobulin

L Palamaro, I Vigliano, G Giardino, E Cirillo, G Aloj, R Romano, C Pignata
Department of Pediatrics, Unit of Immunology, "Federico II" University, Naples, Italy

Key words:Severe combined immunodeficiency. Autoantibody. SCID-like phenotype. T-cell activation defect. Serum inhibitory factor.

Palabras clave: Inmunodeficiencia combinada grave. Autoanticuerpo. Fenotipo IDCG. Defecto de activación de linfocitos T. Factor inhibidor sérico.

Severe combined immunodeficiency (SCID) includes a number of distinct entities that share several clinical hallmarks such as life-threatening infections and intractable diarrhea [1], rapidly leading to growth failure and malnutrition. The clinical course is severe and patients usually die before the second year of life unless a stem cell transplantation is performed. SCID is currently classified into several groups based on the presence or absence of the major lymphocyte cellular components T, B or natural killer (NK) cells, with each group being suggestive of 1 or more genetic causes [1]. However, irrespectively of the individual genetic form, T cell–related functions are constantly abnormal, thus overall compromising a normal, productive

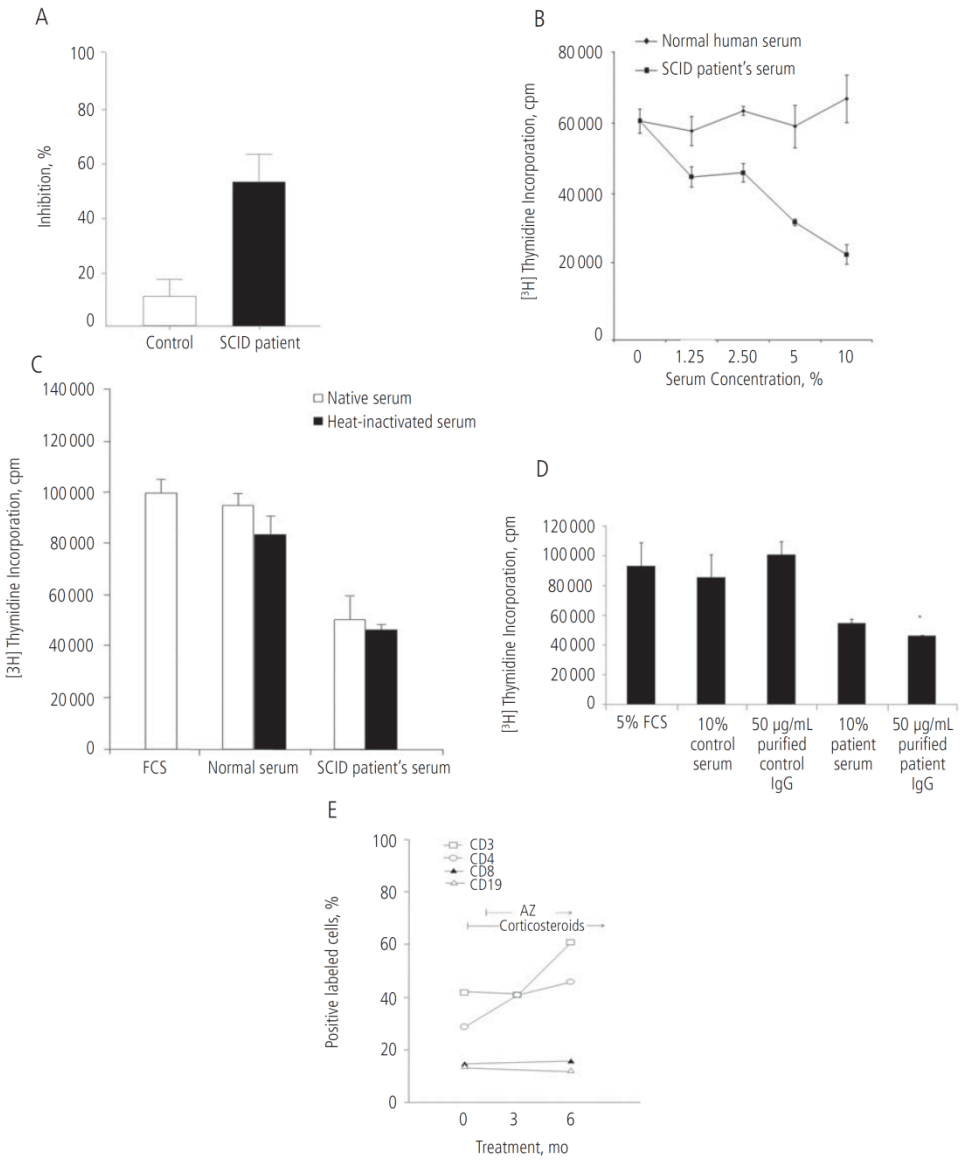

Figure. A, Inhibition (%) of normal peripheral mononuclear cell (PBMC) proliferation after stimulation with phytohemagglutinin (PHA, 8 µg/mL). B, [3H] thymidine incorporation by normal PBMCs stimulated with PHA, (8 µg/mL) and incubated with scalar concentrations (0%, 1.25%, 2.5%, 5%, and 10%) of normal human serum or serum from a patient with severe combined immunodeficiency (SCID). Each point represents the mean (SD), (n=3). C, Effect of native and heat-inactivated serum on proliferative response by normal PBMCs stimulated with PHA (8 µg/mL). Values are expressed as mean (SD) (n=3). D, Inhibition by patient's immunoglobulin (Ig) G of proliferative response by normal PBMCs stimulated with PHA (8 µg/mL). Negative control: fetal calf serum (FCS). Bars show means (SD). *Statistically significant difference ($P<.05$) compared to cultures containing purified control IgG fraction. E, Increase in the percentage of major lymphocyte subsets in patient during treatment with azathioprine (AZ) and corticosteroids. The horizontal lines indicate the period of immunosuppressive treatment with AZ (dotted line) and corticosteroids (solid line). Cpm indicates counts per minute.

immune response in all effector tasks [2]. Severe impairment of T-cell function, however, may also be acquired and induced by viruses, such as the human immunodeficiency virus (HIV) [3]. To date, no autoreactive anti-lymphocyte antibody capable of inducing a SCID-like phenotype has been described.

We report on a 3-year-old patient with a phenocopy of T_{low} B^+ NK^+ SCID. The patient was born at 42 weeks of gestation to healthy, unrelated parents. At 6 months of age the child was hospitalized because of chronic diarrhea, dystrophic features, and febrile seizures. At 8 months of age, the immunological evaluation revealed decreased immunoglobulin (Ig) G serum levels (<47 mg/dL) and normal IgA and IgM. Moreover, the patient had autoimmune hemolytic anemia. At the time of the study, lymphocytes were 3×10^9/L, with 34.5% of $CD3^+$ cells, 25.5% of $CD4^+$ cells, 15.3% of $CD8^+$ cells, 4% of $CD19^+$ cells, and 15% of $CD56^+CD3^-$ cells. Severe lymphocyte functional impairment, in the absence of infection by HIV or any other viruses, was noted. The proliferation assays using phytohemagglutinin (PHA, 8 μg/mL) was performed as previously described [4]. The patient's peripheral blood mononuclear cells (PBMCs) exhibited absent proliferative response to PHA (mean [SD] of 768 [61] counts per minute [cpm] vs 104 649 [21 743] cpm in controls). Over the 3-year follow-up, the patient's PBMC proliferative response ranged between 300 and 8095 cpm. To identify a potential inhibitory factor, the patient's serum was added to PBMCs from 5 healthy controls. A significantly higher inhibitory effect was noted in the patient's serum (53% [10%]) compared to that of the controls (12% [5%]) (Figure A). To define the potency of the inhibitory effect, scalar doses (0%, 1.25%, 2.5%, 5%, and 10%) of the patient's serum and the normal serum were used to produce a dose-response curve. A linear increase in inhibition was observed, with maximum inhibition being reached at the 10% concentration (23 729 [2701] cpm vs 67 050 [6638] cpm in the presence of the control serum) (Figure B). Serum heat inactivation did not abolish the inhibitory effect on the proliferative response to PHA in the control PBMCs, in that the inhibition was 47% (compared to 51% for native serum) (P>.05) (Figure C), thus ruling out a role of the complement in the phenomenon.

To evaluate whether the inhibitory effect in the patient's serum was attributable to an anti-lymphocyte autoantibody, affinity-purified IgG from both the patient's and the control serum was tested for the inhibitory property. Significant inhibition of the proliferative response of normal PHA-stimulated PBMCs was seen in the former but not in the latter case (46 204 [473] cpm vs 100 778 [8988] cpm; P<.05). The inhibition in the presence of the patient's IgG fraction was comparable to that observed with the patient's serum (P>.05) (Figure D).

Thereafter, a progressive decline in $CD4^+$ cells, resulting in a typical lymphocytopenic (0.5×10^9/L) T^- B^+ NK^+ form of SCID, was observed. A similar phenotype is generally due to an impairment in the T-cell differentiation process, resulting in a severe reduction in peripheral T-cell pool size associated with molecular alterations of genes implicated in T-cell ontogeny and function [5]. The prototype of severe T-cell defects, in which NK cells are also often compromised, is related to mutations of the *IL-2Rγ* gene. However, in our case, such gene alterations were ruled out. Two episodes of bronchopneumonia and an interstitial pneumopathy occurred despite intravenous IgG replacement therapy and antibiotic treatment. Autoreactive antibodies toward smooth muscle and red and white blood cells were detected. The patient also developed severely progressive active autoimmune hepatitis, which was diagnosed according to the scoring system established by the International Autoimmune Hepatitis group [6] and treated with azathioprine (1.5 mg/kg/d) and corticosteroids. Diarrhea, autoimmunity, and liver disorders are usually described in relation to T^+ oligoclonal B^- Omenn syndrome [7] and T_{low} B^+ $IL^-7R\alpha$ deficiency [8]. Even though these genetic defects were not ruled out, since at the time of evaluation this information was not available, the clinical and immunological features in our patient are quite different from those seen in these syndromes. At 4 years of age the patient died of disseminated interstitial pneumopathy while the search for an HLA-matched donor was still underway. During this period a paradoxical effect of immunosuppression on cell subsets was noted in that, as depicted in Figure E, there was an increase in $CD3^+$ cells from 42.0% to 60.9% and in $CD4^+$ cells from 28.0% to 46.3%. By contrast, $CD19^+$ and $CD8^+$ cells did not change significantly.

Our data indicate a direct role of the antibody as a negative regulator of T-cell function. However, it is also possible that the inhibitory autoantibody in our patient was the consequence of hyperimmune dysregulation rather than of the T-cell defect, whose genetic alteration still remains to be identified. A similar serum inhibitory effect has also been described in epilepsy, leading to moderate T-cell dysfunction associated with impairment of other immunological cell activities and a decrease in the C4 complement component [9]. However, this functional lymphocyte defect was not as severe as that reported in our patient. Although the functional defect observed in our case may theoretically be related to viral-induced anergy [10], no viral infection was documented and the functional defect was completely differently from that observed in viral-induced anergy.

In conclusion, we have documented a novel pathogenetic mechanism due to an inhibitory anti-lymphocytic autoantibody in a SCID-like phenotype, resulting in total T-cell activation deficiency associated with autoimmunity. This complex phenotype represents a phenocopy of the congenital forms of SCID.

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C Pignata
Department of Pediatrics, Unit of Immunology
"Federico II" University, via S. Pansini 5
80131 Naples, Italy
E-mail: pignata@unina.it

Bronchospasm Induced Selectively by Paracetamol

M Corominas, R Lleonart, E Muñoz
Allergy Unit, Hospital Universitari de Bellvitge, IDIBELL, L'Hospitalet de Llobregat, Spain

Key words: Paracetamol. Bronchospasm. Neurogenic inflammation.

Palabras clave: Paracetamol. Broncoespamo. Inflamación neurogénica.

It is well known that aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) can trigger bronchospasm in susceptible individuals as a result of inhibitory cyclooxygenase (COX) activity. Paracetamol (acetaminophen) is a weak inhibitor of this pathway, but in some patients with NSAID idiosyncrasy, high doses of paracetamol can also provoke bronchospasm.

Although in recent years several epidemiological studies have reported an increased risk of asthma in relation to paracetamol use [1,2], to our knowledge, no cases of selective bronchospasm induced by paracetamol have been published.

We report the case of a patient who developed bronchospasm after paracetamol intake.

A 19-year-old woman was referred to our hospital because she had experienced 8 to 10 episodes of dyspnea within 10 minutes of taking paracetamol over the previous 3 years; this had not occurred with any other NSAIDs (metamizole, ibuprofen). The patient also had a clinical history of rhinitis associated with contact with cats and bronchospasm after exercise.

Skin prick tests to aeroallergens showed positivity for *Alternaria alternata* and for *Olea europaea* pollen. Total immunoglobulin (Ig) E was 41 kU/L and specific IgE to *Alternaria alternata* and cat dander was 3.47 kU/L and 0.23 kU/L, respectively. Spirometry showed mild ventilatory alteration and the bronchodilator response was positive (>12% increase in forced expiratory response in the first second [FEV_1]).

With a diagnosis of rhinitis and asthma with *Alternaria alternata* sensitization, we performed a study with paracetamol with the patient's consent. The prick test (10 mg/mL) and intradermal test (1 mg/mL) to paracetamol were negative. An oral challenge test with paracetamol (100-250-500 mg) and acetylsalicylic acid (ASA) (500 mg) was carried out on different days. Spirometry was performed at baseline and 10 minutes after the intake of placebo and the above-mentioned drugs. The challenge was considered positive when there was a decrease of over 12% in FEV_1 compared to baseline. Salbutamol inhalation was used to evaluate bronchial reversibility after the paracetamol challenge.

Ten minutes after the administration of 500 mg of paracetamol the patient presented bronchospasm and a significant decrease in spirometric values, which returned to baseline after salbutamol inhalation. There were no other systemic or cutaneous symptoms. The administration of 500 mg of ASA did not induce any changes (Table).

To our knowledge this is the first report that confirms the induction of bronchospasm by paracetamol without the involvement of other NSAIDs.

The mechanism by which paracetamol induces bronchospasm in our patient is unclear.

NSAIDs have the ability to induce bronchospasm by inhibiting COX-1, and paracetamol is a weak inhibitor of this enzyme. The fact that high doses of ASA did not induce bronchospasm in the patient makes it highly unlikely that paracetamol acts in this pathway. We also failed to demonstrate specific IgE by means of skin tests.

While a neurogenic mechanism has been implicated as responsible for airway inflammation [3], some authors, based on experimental studies, have more recently suggested that the activation of specific receptors expressed on sensorial neurons could induce inflammation in the airways. In this

§ 2.2 T-helper 1 / T-helper 2 imbalance

The identification of distinct CD4+ T helper cells (Th1 and Th2) exerting peculiar functions and differing on the basis of the production of a unique cytokine profile greatly contributed to our understanding of the intimate mechanism implicated in the different type of host immunity. Th1 cells produce interferon (IFN)-γ and interleukin (IL)-2 and, predominantly, promote cell mediate immune responses, whereas Th2 cells that produce IL-4, IL-5 and IL-13 provide help for some B cell responses as IgG1 and IgE production [115,116]. Overall, an appropriate immune response mostly relies on a well orchestrated Th1/Th2 dichotomy, whose hallmark is based on the capability of the individual subset to work in an autocrine fashion leading to amplify its own cell development and to cross-regulate the other subset development and activity [117,118].

It has been hypothesized that Th1/Th2 imbalance cause elevated IgE serum levels. Moreover, the susceptibility to infections by certain pathogens is associated with low levels of IFN-γ [119]. Thus, alteration of Th1/Th2 homeostasis, also involving further regulatory T cells as Th17, may lead to diseases in humans [117,120]. A Th1 response is implicated under ordinary circumstances in resistance to several intracellular pathogens, but an excessive Th1 response is associated with different autoimmune diseases, as rheumatoid arthritis [121,122], type I diabetes [123] or multiple sclerosis [124]. On the contrary, a Th2 dominated response, usually involved in the response to extracellular pathogens as parasitic or helminths, is associated with allergic disorders and the progression of chronic infections as AIDS [125]. The dimeric cytokine IL-12, produced by B cells and macrophages, plays a pivotal role for the induction of a Th1 response [126]. Its cloned receptor consists of two subunits, IL-12Rb1 and b2, both required for high affinity binding to IL-12 and full cytokine responsiveness [127]. The

receptor is up-regulated during T-cell activation and IL-12Rb2 transcript is selectively expressed in Th1 cells following IL-12 stimulation, while IL-12Rb1 is constitutively expressed in resting cells [128]. The transducing element of the receptor is the IL-12Rb2 chain that functionally interacts with members of the family of STAT, and in particular STAT4. This transcription factor is promptly phosphorylated on tyrosine residues upon receptor triggering [129]. Th1 cells develop in the presence of IL- 12 and STAT4 signaling and secrete mainly IFN-γ [130].

§ 2.2.1 Hyper IgE and IL12R deficiency

Hyper IgE Syndrome (HIES) is a very rare primary immunodeficiency, characterized by the high serum levels of IgE (>2000 IU/ml), recurring staphylococcal skin abscesses and pneumonia with pneumatocele formation. Most cases are sporadic, but both autosomal dominant forms of HIES and autosomal recessive forms have been described. Skeletal symptoms such as hyperextensibility of joints, scoliosis, osteoporosis, and retained primary teeth are associated with the autosomal dominant form. An autosomal recessive disease characterized by severe recurrent viral infections, extreme eosinophilia and devastating neurological complications that are often fatal in childhood, has been described. Patients with the autosomal recessive form appear to be prone to developing autoimmune diseases. HIES usually presents very early in life. Clinical diagnosis has been based on a profile of immunologic and non-immunologic features leading to a composite score. Specific mutations have not been identified in these patients [131].

It has been hypothesized that elevated serum levels of IgE are associated with a Th1/Th2 imbalance.

We investigated, at a functional level, whether an impaired induction of Th1 response occurred in patients with elevated IgE serum levels and whether such abnormalities were correlated with alterations of the IL-12 receptor signaling apparatus. In particular, the activation of STAT4 molecule, which follows IL-12R

triggering, the analysis of gene transcription and membrane assembly of the receptor itself were investigated in allergic children divided on the basis of the amount of serum IgE. We provided evidence of altered IL-12/IL-12R signaling in patients with very high IgE level, suggestive of an impaired Th1 induction.

It is noteworthy that all the abnormalities herein described were observed only in patients with IgE levels higher than 2000 kU/l, which represent only a minority of patients, and not in atopic patients with an ordinary increase of IgE levels. The cut off of 2000 kU/l is generally assumed as a presumptive sign to select patients at risk of being affected by HIES. However, this syndrome was excluded in our patients by the absence of the typical clinical and immunological features [131]. In particular, no recurrent skin infections, facial, skeletal and dentition anomalies were observed.

Additionally, we described a child with recurrent bronchopneumonia associated with very high serum IgE levels, who exhibited a profound impairment of the Th1 generation associated with a variation in the exon 5 of the IL-12R β1 gene (R156H) that exerts a summatory effect, as a genetic cofactor, along with an additional and still unidentified molecular alteration of the IL-12R pathway. Thus, in children with severe and recurrent infection, the functional and/or genetic alterations of the molecular mechanisms governing Th1/Th2 homeostasis might be responsible for an atypical immunodeficiency and, therefore, should be investigated in these patients

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Altered signaling through IL-12 receptor in children with very high serum IgE levels

Anna Fusco, Ilaria Vigliano, Loredana Palamaro, Emilia Cirillo, Giuseppina Aloj, Giovanna Piscopo, Giuliana Giardino, Claudio Pignata *

Department of Pediatrics, "Federico II" University, Naples, Italy

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ABSTRACT

An alteration of Th1/Th2 homeostasis may lead to diseases in humans. In this study, we investigated whether an impaired IL-12R signaling occurred in children with elevated serum IgE levels divided on the basis of the IgE levels (group A: >2000 kU/l; group B: <2000 kU/l). We evaluated the integrity of the IL-12R signaling through the analysis of phosphorylation/activation of STAT4, and mRNA expression and membrane assembly of the receptor chains. At a functional level, a proliferative defect of lymphocytes from group A patients was observed. In these patients, an abnormal IL-12R signaling was documented, and this finding was associated with abnormal expression of the IL-12Rβ2 chain. Our data indicate that in patients with very high IgE levels the generation of Th1 response is impaired, and that this abnormality associates with abnormal IL-12R signaling.

1. Introduction

The identification of distinct $CD4^+$ T helper cells (Th1 and Th2) exerting peculiar functions and differing on the basis of the production of a unique cytokine profile greatly contributed to our understanding of the intimate mechanism implicated in the different type of host immunity. Th1 cells produce interferon (IFN)-γ and interleukin (IL)-2 and, predominantly, promote cell-mediate immune responses, whereas Th2 cells that produce IL-4, IL-5 and IL-13 provide help for some B cell responses as IgG1 and IgE production [1,2]. Overall, an appropriate immune response mostly relies on a well orchestrated Th1/Th2 dichotomy, whose hallmark is based on the capability of the individual subset to work in an autocrine fashion leading to amplify its own cell development and to cross-regulate the other subset development and activity [3,4]. It has been hypothesized that elevated serum levels of IgE are associated with a Th1/Th2 imbalance. Moreover, the susceptibility to infections by certain pathogens is associated with low levels of IFN-γ [5]. Thus, alteration of Th1/Th2 homeostasis, also involving further regulatory T cells as Th17, may lead to diseases in humans [3,6]. A Th1 response is implicated under ordinary circumstances in resistance to several intracellular pathogens, but an excessive Th1 response is associated with different autoimmune diseases, as rheumatoid arthritis [7,8], type I diabetes [9] or multiple sclerosis [10]. On the contrary, a Th2 dominated response, usually involved in the response to extracellular pathogens as parasitic or helminths, is associated with allergic disorders and the progression of chronic infections as AIDS [11].

The dimeric cytokine IL-12, produced by B cells and macrophages, plays a pivotal role for the induction of a Th1 response [12]. Its cloned receptor consists of two subunits, IL-12Rβ1 and β2, both required for high affinity binding to IL-12 and full cytokine responsiveness [13]. The receptor is up-regulated during T-cell activation and IL-12Rβ2 transcript is selectively expressed in Th1 cells following IL-12 stimulation, while IL-12Rβ1 is constitutively expressed in resting cells [14]. The transducing element of the receptor is the IL-12Rβ2 chain that functionally interacts with members of the family of Signal Transducers and Activators of Transcription (STAT), and in particular STAT4 [15]. This transcription factor is promptly phosphorylated on tyrosine residues upon receptor triggering [15]. Th1 cells develop in the presence of IL-12 and STAT4 signaling and secrete mainly IFN-γ [16]. Moreover, experimental evidence using the knock-out technology supports the concept that IL-12R/STAT signaling pathway plays a role for the induction of a Th1 response [17,18].

The aims of our study were to investigate at a functional level whether an impaired induction of Th1 response occurred in patients with elevated IgE serum levels, and whether such abnormalities were correlated with alterations of the IL-12 receptor signaling apparatus. In particular, the activation of STAT4 molecule that follows IL-12R triggering, and the analysis of gene transcription and membrane assembly of the receptor itself were investigated in allergic children divided on the basis of the amount of serum IgE.

* Corresponding author. Address: Department of Pediatrics, Unit of Immunology, "Federico II" University, via S. Pansini, 5-80131 Naples, Italy. Fax: +39 081 545 1278.
E-mail address: pignata@unina.it (C. Pignata).

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2. Materials and methods

2.1. Subjects

Twenty patients with elevated IgE levels and history of allergy were enrolled into the study. Sixteen patients were affected by asthma, 3 of them also by rhinitis, and 4 had a history of atopic dermatitis. The patients divided in two subgroups on the basis of IgE levels: group A consisted of 10 patients, 10 males, range of age 5–15 years, with very high serum IgE levels (>2000 kU/l, range 2152–5000 kU/l); group B consisted of 10 patients, 9 males, range of age 6–15 years, with high serum IgE levels (IgE value between the age specific mean ± 2 SD and 2000 kU/l, range 93–1152 kU/l) (Table 1). Twenty healthy controls, 16 males range of age 6–15 years (IgE range 85–100 kU/l), were also studied. Informed consent was obtained when required. All patients enrolled into the study did not receive any treatment, including steroid or non-steroid drugs, in the month before entering into the study. No difference was found between group A and B in either the number per year or the severity of allergic manifestations. In all patients, the clinical features persisted for more than 2 years.

The Hyper-IgE Syndrome (HIES) was excluded by the absence of typical clinical and immunological features according to the clinical score for HIES (Table 1) [19,20]. In particular, no recurrent skin infections, facial, skeletal and dentition anomalies were observed. Other conditions accompanied by elevated serum IgE concentration, including AIDS, helminths and parasitic infections were also excluded by clinical and laboratoristic features.

The study has been approved by the Institutional Review Board.

2.2. Cell culture and proliferation assay

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (Biochrom, Berlin, Germany) density gradient centrifugation by standard procedure and cultured in triplicates (2×10^5/well). Cells were stimulated with phytohaemagglutinin (PHA; 8 μg/ml), concanavalin A (ConA; 8 μg/ml), pokeweed (PWM, 10 μg/ml) (Difco Laboratories, Detroit, MI), phorbol-12-myristate-13-acetate (PMA; 20 ng/ml) and ionomycin (0.5 mM) (Sigma Chemical Co., St. Louis, MO). CD3 cross-linking (CD3 X-L) was performed by precoating tissue culture plates with 1 and 0.1 ng/ml purified anti-CD3 monoclonal antibody (Ortho Diagnostic, Raritan, NJ). To evaluate allogeneic response, patients responder cells (1×10^5) were stimulated with an equal amount of irradiated stimulator cells from controls in a standard one-way mixed lymphocyte reaction assay. Cell mixtures were cultured in 96-well round-bottom microtiter plates (Becton Dickinson, San Jose, CA) for 5 days and harvested 18 h after [^{3}H]thymidine pulsing.

Table 1
Clinical characteristics of patients divided in group A and group B included in the study.

Patients	Gender	Age	Clinical features	HIES score	Serum IgE levels (kU/l)
1	M	6	Asthma	0	<2000
2	M	6	Asthma	1	<2000
3	M	7	Asthma	1	<2000
4	M	9	Asthma, rhinitis	8	<2000
5	M	11	Asthma	4	<2000
6	M	8	Asthma	1	<2000
7	F	15	Asthma	4	<2000
8	M	6	Asthma, rhinitis	8	<2000
9	M	6	Atopic dermatitis	8	<2000
10	M	10	Asthma	0	<2000
11	M	7	Atopic dermatitis	10	<2000
12	M	5	Atopic dermatitis	10	>2000
13	M	5	Asthma	13	>2000
14	M	7	Asthma	13	>2000
15	M	8	Asthma, rhinitis	13	>2000
16	M	10	Asthma	13	>2000
17	M	15	Asthma	10	>2000
18	M	12	Asthma	13	>2000
19	M	5	Atopic dermatitis	10	>2000
20	M	7	Asthma	10	>2000

2.3. Generation of Th1 cell lines

Th1 cell lines were generated by stimulating PBMC with PHA (8 μg/ml) or, in a few experiments, with PHA + IFN-γ (1000 U/ml, ICN, Biomedical, OH) for 72 h in complete tissue culture medium. These cells usually widely express high affinity IL-12R.

2.4. Analysis of STAT4 activation

PHA-induced blasts were made quiescent by 12 h incubation in RPMI supplemented with 2.5% FCS at RT, and further stimulated with rIL-12 (Genetics Institute, Cambridge, MA) at a concentration of 10–100 U/ml for 10 min. After the appropriate stimuli, $3–5 \times 10^6$ cells were lysed in buffer containing 20 mM Tris, pH 8, 10% glycerol, 137 mM NaCl, 1% Nonidet P-40, 10 mmol EDTA, 1 mM phenyl methane sulfonyl fluoride (PMSF), 1 mM sodium orthovanadatum (Na3Vo4), 5 μg/ml leupeptin and 5 μg/ml aprotinin. Proteins were resolved by 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes and then blocked with 5% bovine serum albumin. Immunoblotting was performed by a 2–4 h incubation with anti-STAT4 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Signals were detected using chemiluminescence (ECL system, Amersham, Buckinghamshire, England). The low migration supershifted form of STAT4 indicates the presence of the protein in its activated/phosphorylated form [21]. Densitometric analysis was performed to evaluate the overall amount of the protein and the amount of its supershifted form.

2.5. Membrane expression of β1 and β2 chains of IL-12R on T cells

After washing in PBS, cells were incubated for 20 min sequentially with murine anti-β1 or anti-β2 chain (25 μl) of IL-12R (kindly provided by Dr. Jerome Ritz, Dana Farber Cancer Institute, Boston, MA), IgG1 isotype control Ab, 10 μl FITC-conjugated goat anti-mouse IgG Ab (Becton Dickinson, San Jose, CA), and 5 μl anti-CD4 PE Ab (Becton Dickinson, San Jose, CA). After staining, the expression of IL-12Rβ1 and β2 on CD4$^+$ cells was determined with flow cytometer (Becton Dickinson) by gating on the CD4$^+$ population.

2.6. Analysis of IL-12Rβ2 chain RNA expression

Total cellular RNA was prepared using Trizol reagent method (Sigma Chemical Co., St. Louis, MO); 1 μg of total RNA was reverse transcribed into cDNA using Expand™ Reverse transcriptase according to the manufacturer's protocol (Boehringer Manneheim, Germany). The cDNA was PCR amplified (94 °C, 1 min; 55 °C, 1 min; 72 °C,1 min for 30 cycles) using specific primers for IL-12Rβ2: sense primer GGAGAGATGAGGGACTGGT and antisense primer TCACCAGCAGCTGTCAGAG. Each PCR mixture consisted of 3 μl of cDNA, 1 μl of each primer (concentration from Kathy), 0.2 mM dNTP and 2.5 U of Taq DNA polymerase (Life Technologies Ltd., Paisley, Scotland). These reactions were carried out in a buffer

containing 25 mM $MgCl_2$, 200 mM Tris–HCl and 500 mM KCl. To monitor the amount of RNA, β-actin mRNA expression was used. PCR products were separated in a 1% agarose gel and viewed after ethidium bromide staining.

2.7. Statistical analysis

The significance of differences was evaluated by Wilcoxon rank sum test for unpaired data. All the data were obtained from at least three distinct experiments performed in a 6 months period.

3. Results

3.1. Proliferative responses

Fig. 1A illustrates the proliferative response to CD3 cross-linking (CD3 X-L) performed at optimal antibody concentration, that mimics *in vivo* antigen exposure in patients and controls. Group A patients showed a significantly lower response than controls (mean ± SD: 23,200 ± 6402 *versus* 49,690 ± 4398 cpm in controls, $p < 0.05$). In contrast, patients of group B had a higher proliferative response not significantly different from controls. Similarly, the proliferative response to PHA was lower in group A than in the other groups (mean ± SD: group A, 58,790 ± 11,690 cpm; group B, 106,500 ± 10,800 cpm; controls 93,070 ± 4455 cpm. A *versus* B and A *versus* controls: $p < 0.01$). No difference was found in the proliferative assays with the other stimuli. As depicted in Fig. 1B, the allogeneic response was comparable in the three groups.

3.2. Analysis of STAT4 tyrosine phosphorylation/activation

IL-12/IL-12 receptor signaling plays a crucial role in Th1 induction. To evaluate whether the low response to CD3 X-L associated with a normal allogeneic response was due to an impaired Th1 generation, we next investigated IL-12R signaling by analyzing supershift of the transcription factor STAT4, that promptly occurs after receptor triggering by its own cytokine and indicates protein tyrosine phosphorylation of the molecule [21]. Fig. 2A shows a representative experiment out of six performed indicating that in controls rIL-12 stimulation for 10 min of PHA-induced cell lines induces STAT4 supershift, due to the appearance of a slower migrating form representing the phosphorylated molecule. By contrast, in all patients of group A there was no supershift of STAT4, and the molecule appeared as a single form of 84 kDa. Fig. 2B illustrates the densitometric analysis representing the overall amount of STAT4 and the amount of its supershifted form. The protein was expressed in patients and controls in a comparable amount. IL-12 stimulation induced the supershift only in control cells and not in patient cells. Fig. 3A shows that IL-12 stimulation of cell

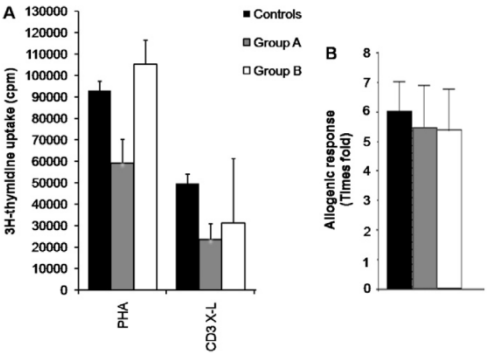

Fig. 1. Proliferative responses in patients and controls. Patients were divided on the basis of IgE levels (group A, $n = 10$, IgE >2000 kU/l; group B, $n = 10$, IgE value between the age specific mean ± 2 SD and 2000 kU/l; controls, $n = 20$). (A) Proliferative response to PHA (8 μg/ml) and CD3 cross-linking (CD3 X-L), performed by precoating tissue culture plates with 1 and 0.1 ng/ml purified anti-CD3 monoclonal antibody. Each column represents the mean value ± SD. (B) Proliferative response to allogeneic stimuli. Results are expressed as the mean value ± SD and indicate the times fold increase over the background.

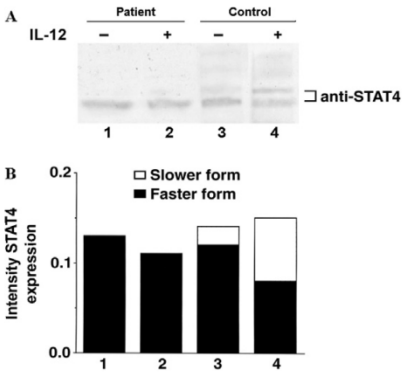

Fig. 2. Analysis by immunoblot of STAT4 protein in controls and patients with very high IgE levels (>2000 kU/l). (A) Representative experiment, out of 6, showing that rIL-12 stimulation induces in controls the appearance of a slow migrating phosphorylated form of the protein, whereas in patients only the 84 kDa protein is evident. PBMC from a patient (lanes 1 and 2) or control (lanes 3 and 4) were incubated with PHA for 72 h, and then further stimulated with rIL-12 for 10 min (lanes 2 and 4) or medium alone (lanes 1 and 3). (B) Measure by densitometric analysis of the amount of the STAT4 protein. White region of each column indicates the slower hyperphosphorylated form of the protein, the black areas indicate the faster form. Each column is referred to the corresponding lane of the panel A.

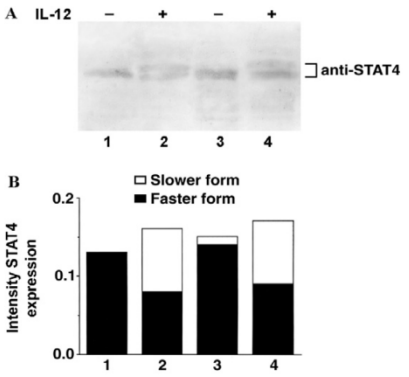

Fig. 3. Analysis by immunoblot of STAT4 protein in controls and patients with IgE values between the age specific mean ± 2 SD and 2000 kU/l. (A) Representative experiment, out of 3, showing that rIL-12 stimulation induces both in control and patient the appearance of a slower form of STAT4. PBMC were processed as indicated in Fig. 2 and Section 2. Lanes 1 and 2, patient; lanes 3 and 4, control. Cells were stimulated with rIL-12 for 10 min (lanes 2 and 4) or medium alone (lanes 1 and 3). (B) Measure by densitometric analysis of the amount of the STAT4 protein. White region of each column indicates the slower hyperphosphorylated form of the protein; the black areas indicate the faster form. Each column is referred to the corresponding lane of the panel A.

lines obtained from patients of group B, induced the appearance of the slower supershifted form of STAT4 both in controls and patients to a similar extent.

3.3. IL-12R expression on T cells

The high affinity IL-12 receptor consists of β1 and β2 chains, the latter being up-regulated during cell activation and selectively expressed on Th1 cells. To determine whether the failure of STAT4 phosphorylation was due to decreased expression of IL-12 receptor, we analyzed the surface expression of IL-12R in T cell lines induced in the presence of PHA. The expression of both β1 and β2 chains was lower in group A than in the other groups. IL-12Rβ1 values, expressed as mean percentage of positively stained cells ± SD, were as follows: group A, 35.96 ± 7.3%; group B, 53.8 ± 6.6%; controls, 51.7 ± 6.1%. Similarly, a lower up-regulation of β2 chain in group A was observed as depicted in Fig. 4A. Mean percentage values ± SD of IL-12Rβ2 expression were 16.5 ± 3.0% in group A; 28.8 ± 3.7% in group B; 28.9 ± 1.6% in controls (A *versus* B and controls: $p < 0.05$). The mean fluorescence intensity was lower in the group A than in the other groups (Fig. 4B), differently from β1 whose intensity was comparable in the three groups (data not shown).

3.4. IL-12Rβ2 mRNA expression

We next analyzed the mRNA expression of IL-12Rβ2 chain in group A, where no STAT4 tyrosine phosphorylation was observed. The expression of the IL-12Rβ2 transcript in all experiment performed was different between patients and controls. In three experiments there was no induction at all of β2 transcript after of 18, 36 and 48 h PHA stimulation, as illustrated in a representative experiment in Fig. 5. Furthermore, no effect of IFN-γ was noted. These data were confirmed by five distinct experiments. In two cases there was mRNA expression, but in one case it was delayed appearing only after 48 h PHA stimulation, even though it

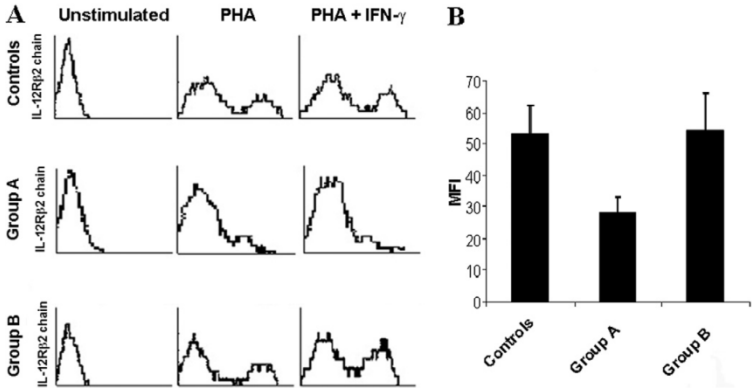

Fig. 4. Membrane expression of β2 chain of IL-12R on T cells. IL-12Rβ2 membrane expression on resting or T-cell blasts, induced by stimulation with PHA for 72 h in the absence or presence of IFN-γ, in controls and patients divided in two groups on the basis of IgE levels as indicated in Section 2. Dual colour fluorescence using FITC-conjugated anti-β2 and PE-conjugated anti-CD4 was performed. (A) Shows a representative experiment indicating the lower up-regulation of β2 chain in group A. The mean fluorescence intensity in the three groups is shown in (B). Each column represents the mean value ± SD. The intensity was lower in the patients of group A than in the other groups.

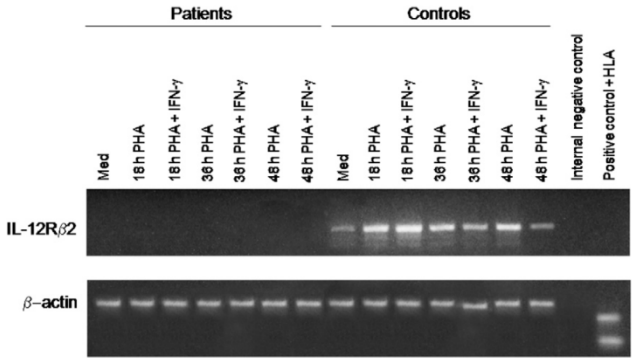

Fig. 5. mRNA expression of IL-12Rβ2 chain in controls and group A patients (IgE levels: >2000 kU/l). Representative experiment showing that in controls, β2 chain mRNA expression increased after 18 h of PHA stimulation. Lanes 1 and 8: freshly isolated PBMC. T-cell blasts were generated by 18, 36 and 48 h of PHA stimulation. IFN-γ upregulated β2 chain mRNA expression after short term PHA stimulation, but it was ineffective during longer stimulations. In group A there was no induction at all of β2 transcript. Furthermore, no effect of IFN-γ was noted.

was also slightly appreciable after 36 h stimulation in the presence of IFN-γ. In the other case a faint signal was appreciable after 18 h of PHA stimulation, but it rapidly disappeared.

4. Discussion

In this study, we provided evidence of altered IL-12/IL-12R signaling in patients with very high IgE level, suggestive of an impaired Th1 induction. In particular, defective supershift of the STAT4 molecule following rIL-12 stimulation of T-cell blasts was documented. Supershift of this molecule indicates its phosphorylation [21]. This finding was associated with a T-lymphocyte functional derangement characterized by low proliferative response to stimulations via TCR/CD3 complex, but with a preserved allogeneic response. The discrepancy between mitogenic and allogeneic stimuli in inducing cell proliferation has already been documented in mice in which the gene coding for 40 kDa subunit of IL-12 has been disrupted [17]. These mice are not able to generate most of the Th1 responses, including IFN-γ production and delayed type hypersensitivity response *in vivo*, but cytolytic response elicited by allogeneic stimuli was preserved, thus suggesting that the allogeneic response is dependent on a wider array of cytokines influences. Further evidence on the role of the IL-12/IL-12R signaling apparatus on the induction of Th1 responses comes from the functional studies on mice lacking STAT4 molecule, that represents a central signaling protein involved in IL-12R signaling [22]. Although there is evidence suggesting that the development of Th1 type responses may also take place in a STAT4 independent fashion [23,24], the STAT4 knock-out experimental model underlines the importance of the integrity of the IL-12/IL-12R signaling for the generation of a proper Th1 type response. Again, $STAT4^{-/-}$ mice exhibit a propensity to generate Th2 type cells [22]. It is noteworthy that all the abnormalities herein described were observed only in patients with IgE levels higher than 2000 kU/l, which represent only a minority of patients, and not in atopic patients with an ordinary increase of IgE levels. The cut-off of 2000 kU/l is generally assumed as a presumptive sign to select patients at risk of being affected by Hyper-IgE Syndrome (HIES). However, this syndrome was excluded in our patients by the absence of the typical clinical and immunological features [19,20]. In particular, no recurrent skin infections, facial, skeletal and dentition anomalies were observed.

The link between viral and bacterial infections and the pathogenesis of allergic asthma has represented for years an appealing area of clinical investigation, which is currently expanding in parallel with the worldwide increase of childhood asthma prevalence [25]. Longitudinal studies indicate that respiratory tract infections may predispose children to asthma [26]. Persistent wheezing seems to be related to increased IgE levels and eosinophils at the time of the first respiratory infection, thus suggesting that infections may trigger asthma attacks in already predisposed subjects [27,28]. However, in contrast to this, it has been shown that early infections may protect against the subsequent development of an atopic phenotype [29]. This hypothesis is also supported by the recently documented inhibitory effect on Th2 cell functions of Th1-released proinflammatory cytokines [30]. Public health measures, as hygiene programs to reduce foodstuffs contamination, active immunization programs, a better pharmacological control of infections, may certainly have contributed in limiting the immune system challenge by infectious agents in early childhood, even though hygiene hypothesis should be revisited in the light of recent data on the role of Toll like receptors and regulatory mechanisms [31]. However, in our study it should be noted that there were not striking differences between the three groups of subjects with regards to vaccination program, social habits and the number or severity of infections in the clinical history that preceded the appearance of allergic disorders.

However, it should be noted that abnormalities of IL-12 signaling pathway is not sufficient *per se* to cause an allergic disease in children, in that the prevalence of asthma, eczema and rhinoconjunctivitis is similar in patients with or without genetic alteration of IFN-γ or IL-12Rβ1 [32]. Our data could imply a link between infections and allergy in children, even though this matter is still under debate and no conclusive demonstration is available [31]. In the light of this consideration, a better understanding of the molecular mechanisms governing Th1/Th2 homeostasis may help ameliorate the overall management of these patients. In fact, there is evidence that patients with severe forms of allergic manifestations are more susceptible to respiratory infections, and vice versa infections may trigger acute episodes of asthma [25]. A defective induction of a Th1 response in patients with very high IgE levels may lead to a higher risk of infections, thus worsening the overall outcome.

Overall, our results indicate that children with very high serum IgE levels have functional and biochemical signs of an altered IL-12/IL-12 receptor signaling network.

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CASE REPORT Open Access

Interleukin 12 receptor deficiency in a child with recurrent bronchopneumonia and very high IgE levels

Loredana Palamaro[1], Giuliana Giardino[1], Francesca Santamaria[1], Rosa Romano[1], Anna Fusco[1], Silvia Montella[1], Mariacarolina Salerno[1], Matilde Valeria Ursini[2] and Claudio Pignata[1,3*]

Abstract

Interleukin-12 (IL-12) is involved in cellular immune responses against intracellular pathogens by promoting the generation of T naive in T helper 1 (Th1) cells and by increasing interferon-gamma (IFN-gamma) production from T and natural killer (NK) cells. A defective induction of a Th1 response may lead to a higher risk of infections, and, in particular, infections due to typical and atypical *Mycobacteria*. We report on the case of a girl with suffering from recurrent bronchopneumonia associated with very high serum IgE levels, who exhibited a profound impairment of the Th1 generation associated with a novel mutation in the exon 5 of the IL-12R β1 gene (R156H). Our data suggest that in children with severe and recurrent infections, even in the absence of a mycobacterial infection, functional and/or genetic alterations of the molecular mechanisms governing Th1/Th2 homeostasis might be responsible for an atypical immunodeficiency and, therefore, should be investigated in these patients.

Keywords: Immunodeficiency, IL-12/IL-12 receptor, Recurrent pneumonia

Background

Primary congenital immunodeficiencies encompass a wide spectrum of distinct clinical entities, which differ in either pathogenetic mechanism or clinical features. Recently, several novel syndromes with unusual phenotypes have been described [1,2]. However, in a number of patients suffering from severe and sometimes life-threatening infections, in which an immunological disorder is suspected, the underlying genetic defect responsible for the immunodeficiency still remains to be elucidated [3]. Recently, a higher susceptibility to intracellular pathogens and, in particular, atypical mycobacterial and salmonella infections has been described in patients with genetic alterations of the IL-12 receptor (IL-12R) [4-9]. IL-12 stimulates cellular immune responses against intracellular pathogens by promoting the generation of T naive in T helper 1 (Th1) cells and by increasing interferon-gamma (IFN-gamma) production from T and natural killer (NK) cells. Induction of a Th1 response and cell cycle progression mostly relies on the expression of a high affinity IL-12R, consisting of β1 and β2 chains [10-15]. A few genetic alterations of β1 chain have already been reported in patients suffering of mycobacterial infections [5,6].

Case presentation

A 8-year-old girl was referred to the our Department because of a history of recurrent pneumonia (4 episodes over 2 years). At the age of 4 years and 8 months she had suffered from the first episode of middle lobar bronchopneumonia requiring hospitalization. In that occasion the total IgE serum levels were 3350 kU/l. One month later, she was hospitalized for a second bronchopneumonia episode interesting both lungs followed by persistent cough for more than a month. These episodes were responsive to antibiotic therapy. Subsequently, she had suffered from 2 additional bronchopneumonia episodes in distinct lung area, successfully treated with parenteral antibiotic therapy. Conventional x-ray and high resolution computed tomography of the chest revealed multiple focal consolidations in both lungs, confirmed

* Correspondence: pignata@unina.it
[1]Department of Pediatrics, "Federico II" University, Naples, Italy
[3]Unit of Immunology, Department of Pediatrics, "Federico II" University, Via S. Pansini 5-80131, Naples 80127, Italy
Full list of author information is available at the end of the article

by magnetic resonance imaging [16]. Acid resistant bacillus was not found in the sputum examination. In one occasion, *Haemophilus influenzae* was isolated on sputum culture. Weight and height growth was in the normal range. No infections in other organs were reported. The routine immunological evaluation revealed normal IgG and IgA, but very high serum IgE levels (> 2000 kU/l), confirmed in several occasions during the 2 years follow-up. Specific IgE toward Dermatophagoides farinae and pteronyss, olive, herb vitriol and Parietaria judaica were detected. Prick test were positive (ponf > 0.3 × 0.4 cm) for Dermatophagoides farinae and pteronyss, hair of dog, Parietaria and olive, thus confirming a multiple sensitivity. The patient showed a proper antibody specific response as demonstrated by the presence of IgG antibody serum levels, tested by immuno-enzymatic method, against B-hepatitis, parotitis and German measles viruses. Serum IgG, IgA e IgM levels were always in the normal range. The immunophenotype valuation revealed normal number and percentage of the lymphocyte subpopulations studied (Table 1).

The patient's family history was notable for the presence of allergic disorders in both lineages. In particular, her mother and grandmother had a history of allergic rhinitis, while her father had urticaria. A maternal aunt died at 2 years of age by whooping-cough and a maternal uncle died at 16 months by a severe not better specified respiratory infection. A paternal aunt and her daughter had a history of allergic rhinitis.

We first determined the proliferative response of PBMC to CD3 cross-linking, that mimics in vivo antigen exposure, performed at optimal (1 ng/ml) or suboptimal (0.3 ng/ml) antibody concentration. The proliferation at the maximal dosage was significantly lower in the patient than in the controls (mean ± SE were: 800 ± 68 cpm and 29500 ± 3000 cpm in the proband and controls, respectively). Since a proper immune response to pathogens requires a Th1 induction and this process determines up-regulation of the expression of the IL-12R β2 chain, we evaluated mRNA expression of this molecule after mitogen stimulation of PBMC in vitro. No expression of IL-12R β2 transcript was found in patient's cells (Figure 1A), differently from the controls. IL-12R β1 was expressed at normal levels (Figure 1B).

At molecular level, gene sequencing of IL-12R β2 gene revealed a missense mutation (G to A) at nucleotide 531 in the exon 5 in heterozygosity, resulting in the substitution of arginine (CGT) with histidine (CAT) in the extracellular domain of the receptor at the same aminoacid position 156 (designed R156H) (Figure 2). The mutation was not a polymorphism since was not found in 100 chromosomes from unrelated individuals. This G to A transition creates a new restriction site for NdeI enzyme (data not shown).

Table 1 Immunonological parameters

Lymphocyte subpopulations	%	n/mm^3
CD3	77	2.956
CD3DR	4.2	161.28
CD4	39.3	1509
CD8	30.2	1159
CD19	11.4	437.76
CD56	3.4	130.56
CD4-CD8-TCR α/β+	2.3	88.32
CD4-CD8-TCR g/d+	2.2	84.48
Specific antibody responses	**IgG**	**IgM**
B-hepatitis virus	Present	Absent
Parotidis virus	Present	Absent
German measles virus	Present	Absent
Proliferative response to mitogens stimulation	**Patient (mean ± SE)**	**Control (mean ± SE)**
PMA + iono	22458 ± 11013	32159 ± 27858
CD3 X-L	800 ± 68	29500 ± 3000

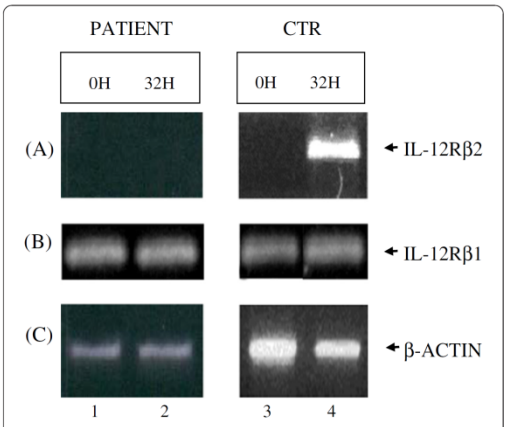

Figure 1 mRNA expression of IL-12R β2 chain after mitogen stimulation of PBMC in vitro. (**A**) No expression of IL-12R β2 transcript was found in patient's cells, differently from the controls. (**B**) IL-12R β1 was expressed at normal levels.

Discussion

The case here reported indicates that alterations of the induction of a proper Th1 response may be associated with an atypical immunodeficiency characterized by high susceptibility to infections. The functional response of lymphocytes to IL-12 depends on the expression of a high affinity IL-12 receptor on Th1 and NK cells. The high affinity receptor for IL-12 consists of two subunits,

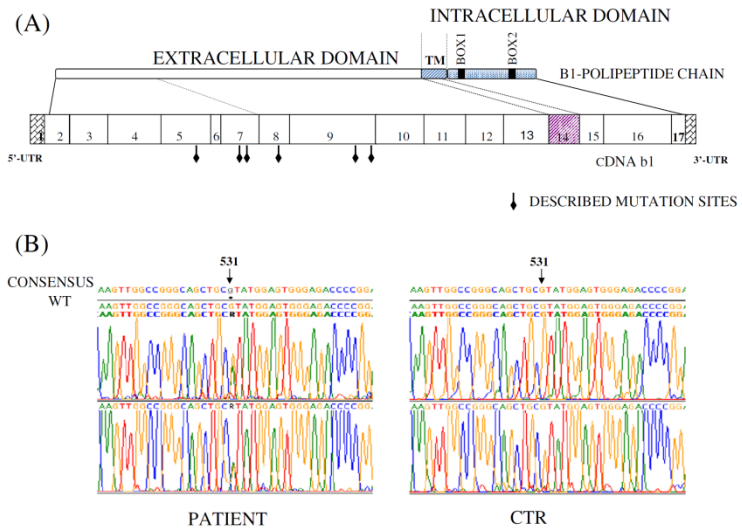

Figure 2 gene sequencing of IL-12R β2 gene. At molecular level, gene sequencing of IL-12R β2 gene revealed a missense mutation (G to A) at nucleotide 531 in the exon 5 in heterozygosity, resulting in the substitution of arginine (CGT) with histidine (CAT) in the extracellular domain of the receptor at the same aminoacid position 156 (designed R156H).

β1 and β2, closely related to the cytokine receptor glycoprotein (gp) 130 [11,17]. The complete IL-12R is thought to be associated with the development, being expressed on human naive T cells during differentiation to Th1 but not to Th2. Therefore, the expression of these molecules is generally considered as a marker of Th1 dominated response [11-13,16]. Th1 cells produce IFN-gamma and IL-2 and, predominantly, promote cell mediate immune responses against intracellular pathogens [18,19]. In a previous study, we provided evidence of altered IL-12/IL-12R signaling in patients with very high IgE levels, suggestive of an impaired Th1 induction [20]. A defective induction of a Th1 response in patients may lead to a higher risk of infections, thus worsening the overall outcome of patients with very high IgE levels. In the case herein described a genetic alteration of the IL-12R β1 has been found in heterozygosity. Whether this alteration is really responsible for the phenotype remains to be definitively demonstrated with further molecular and functional studies. However, it should be noted that patients with homozygous alterations of the same gene have already been reported, being affected with a more severe clinical phenotype and selective susceptibility to mycobacterial infections [5,6]. Based on this clinical observation, we suggest that a better understanding of the molecular mechanisms governing Th1/Th2 homeostasis may help recognize novel clinical phenotypes of atypical immunodeficiencies.

Consent

Written informed consent was obtained from the parents of the patient for publication of this Case report and any accompanying images.

Abbreviations

IL-12: Interleukin-12; Th1: T helper 1; IFN-gamma: Interferon-gamma; NK: Natural Killer.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LP has made substantial contributions to conception and design, has been involved in drafting the manuscript, and has given final approval of the version to be published. GG has made substantial contributions to conception and design, has been involved in drafting the manuscript, and has given final approval of the version to be published. FS has made substantial contributions to conception and design, has been involved in revising the manuscript critically for important intellectual content, and has given final approval of the version to be published. RR and AF has made substantial contributions to acquisition of data, has been involved in drafting the manuscript, and has given final approval of the version to be published. SM has made substantial contributions to acquisition of data, has been involved in revising the manuscript critically for important intellectual content, and has given final approval of the version to be published. MVU has made substantial contributions to conception and design and analysis and interpretation of data, has been involved in revising the manuscript critically for important intellectual content, and has given final approval of the version to be published. CP has made substantial contributions to conception and design and analysis and interpretation of data, has been involved in drafting the manuscript and revising it critically for important intellectual content, and has given final approval of the version to be published.

Author details
[1]Department of Pediatrics, "Federico II" University, Naples, Italy. [2]International Institute of Genetics and Biophysics, CNR, Naples, Italy. [3]Unit of Immunology, Department of Pediatrics, "Federico II" University, Via S. Pansini 5-80131, Naples 80127, Italy.

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§2.3 Conclusive remarks

Recent evidence indicates that systemic autoimmunity and immunodeficiency can be strictly linked. Molecular mechanisms involved in central tolerance, along with those in peripheral tolerance, play a crucial role in the establishment and maintenance of immune self-tolerance, preventing autoimmunity and promoting the proper function of immune system. The discovery of genetic diseases caused by alterations of genes implicated in the tolerance mechanisms enormously contributed to our understanding of the molecular basis of human autoimmune disorders, generally and appropriately considered as multifactorial diseases.

Moreover, a better understanding of the molecular mechanisms governing Th1/Th2 homeostasis may help ameliorate the overall management of the patients [132].

CHAPTER III

"Alteration of immune system genes: cancer predisposition and neurodegeneration"

The immune system has the greatest potential for the specific destruction of tumors with no toxicity to normal tissue and for long-term memory that can prevent cancer recurrence. The last 30 years of immuno-oncology research have provided solid evidence that tumours are recognized by the immune system and their development can be stopped or controlled long term through a process known as immune surveillance [133,134].

In many cancers, however, malignant progression is accompanied by profound immune suppression that interferes with an effective antitumour response and tumour elimination [135]. It has become clear that the suppression comes from the ability of tumours to subvert normal immune regulation to their advantage. The tumour microenvironment can prevent the expansion of tumour antigen-specific helper and cytotoxic T cells and, instead, promote the production of pro-inflammatory cytokines and other factors, leading to the accumulation of suppressive cell populations that inhibit instead of promote immunity.

A strong predisposition for cancer development has been reported for some types of PIDs [6,7], such as ataxia-telangiectasia (AT), common variable immunodeficiency (CVID), Wiskott-Aldrich Syndrome (WAS), SCID, selective IgA deficiency, DNA repair deficiencies and Hyper IgM Syndromes. The predisposition to cancer could be due to immunodeficiency itself, as tumor immune surveillance becomes impaired and infections by potentially oncogenic viruses are less likely to be dealt with effectively [136]. The main type of malignancy in PIDs is non Hodgkin lymphoma, which account with a frequency of 60 %, followed by Hodgkin lymphoma (23%) and leukemia (6%) [8]. All these types of malignancies develop more frequently in extranodal site, particularly in the central nervous system and gastrointestinal tract, and derive from malignant B cell proliferation [137]. However, the type of malignancy

depends on the specific PID, the age and the infective clinical history of the patients. The genetic defect, underlying the immunodeficiency, may play a direct role in cancer pathogenesis either facilitating chronic infection, especially with oncogenic viruses such as Epstein Barr virus (EBV), or through genetic mutations in DNA double-strand breaks repair systems, which lead to accumulation of mutations that promote tumorogenesis [9].

In SCID due to mutations of genes involved in VDJ recombination, the genetic defect plays a direct role in the carcinogenesis. These genes, in fact, encode proteins, such as Artemis and DNA ligase IV, involved in DNA double-strand breaks repair. Impairment of this process lead to accumulation of gene mutations, worsened by radiosensitivity, which accelerate the malignant transformation of lymphocytes [138-140]. The same pathogenetic mechanisms can be observed in Atassia-Teleangectasia. AT represent the primary immunodeficiency with the highest risk of malignancy [141]. ATM who is a member of the phosphatidyl inositol kinase molecule family, normally acts as a sensor of double-stranded DNA breakage. In this context the predisposition to leukemia is connected to excessive production of translocations [7].

Hyper-IgM syndromes are caused by mutations in the genes encoding for the CD40/CD40L interaction pathway leading to defects in the class switch recombination. X-linked hyper-IgM syndrome is associated with a highest risk to develop carcinomas of the liver, pancreas, biliary tract and associated neuroectodermal endocrine cells [142]. The chronic inflammatory stimulation due to defective clearance of cryptosporidia plays an important role in the carcinogenesis also in these syndromes in which carcinoma are often preceded by chronich epatitis and cholecystitis [143].

The role of γc, IL-7R and JAK3 in tumor development further cement the relation between immunity and cancer even though in SCIDs associated with mutations in these genes cannot be observed an increased cancer susceptibility.

§3.1 The role of γc in endocrine system and cancerogenesis

IL-2RG encodes the common cytokine γc of the IL-2 receptor. The γc gene, localized to chromosome Xq13, encodes a transmembrane protein which is a transducing element shared by the cytokine receptor superfamily, including IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. [144,145]. Mutations in this gene are responsible for the X-linked Severe Combined Immunodeficiency (X-SCID), characterized by the complete absence of both T and NK lymphocytes, whereas B cell number is normal.

The receptors containing γc exert prominent mitogenic effects and play an important role in several immunological functions and in supporting cell survival. The γc is involved also in growth hormone receptor (GH-R) signaling. The impairment of various GH induced events in patients affected with severe combined immunodeficiencies due to γc defects suggests a potential functional interaction between GH-R pathways and γc, indicating a further link between endocrine and immune system with potential implication of the molecule in the cell cycle progression and control. Moreover, evidence indicates that the GH/IGF-I axis has a role in the development of cancer through the regulation of cell proliferation, differentiation and apoptosis [146]. Even though IGF-I is mitogenic per se and exerts an important antiapoptotic effect the GH-R signaling apparatus also involves potent mitogenic molecules such as γc and signal transducers and activators of transcription (STAT) that play a role in the cell proliferation and, in general, in cell homeostasis [147-149]. Furthermore, as been shown that both GH induced and spontaneous cell cycle progression and cell growth are strongly dependent on the amount of γc expression.

Of note, STATs play important roles in oncogenesis by up-regulation of genes encoding apoptosis inhibitors and cell cycle regulators, namely oncogenes, such as Bcl-xL, Mcl-1, cyclins D1/D2, and c-Myc. It has been also documented a constitutive activation of STAT3 or STAT5 in tumor cells resistant to chemotherapeutic agents, that exert their effect on cell apoptosis machinery.

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Review

γ Chain transducing element: A shared pathway between endocrine and immune system

Ilaria Vigliano, Anna Fusco, Loredana Palamaro, Giuseppina Aloj, Emilia Cirillo, Maria Carolina Salerno, Claudio Pignata *

Department of Pediatrics, Unit of Immunology, 'Federico II' University, via S. Pansini 5-80131, Naples, Italy

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ABSTRACT

Several molecules, involved in the intracellular communication network, have been identified as the cause of primary immunodeficiencies. In most cases, these molecules are exclusively expressed in hematopoietic cells, being involved in cell development and/or functionality of terminal differentiated cells of immune system. In the case of γc, the abundance of the protein suggests a potential pleiotropic effect of the molecule. Immune and endocrine systems participate to an integrated network of soluble mediators that communicate and coordinate responsive cells to achieve effector functions in an appropriate fashion. It has been demonstrated a novel dependence of GH signaling on the common cytokines receptor γc in certain cell types, supporting the hypothesis of an interplay between endocrine and immune system. The evidence that different receptors share a few molecules may certainly lead to a better knowledge on the mechanism of coordination and integration of several pathways implicated in the control of cell growth and proliferation under physiological or pathogenic conditions. This review focuses on the γc as a common transducing element shared between several cytokines and growth hormone receptors, indicating a further functional link between endocrine and immune system.

1. Introduction

The common γ-chain (γc) gene localized to chromosome Xq13 encodes a transmembrane protein which is a transducing element shared by the receptors for interleukin (IL)-2, IL-4, IL-7, IL-9, IL-15 and IL-21 [1]. Deficiency in the expression or function of the γc causes the X-linked severe combined immunodeficiency (X-SCID) [2]. SCIDs are a group of rare primary immunodeficiencies (PID), distinct in either the clinical and immunological phenotype or the pathogenetic mechanism. X-SCID is the most common form of SCID, accounting for approximately half of the cases of SCID and is the main form of $T^-B^+NK^-$, in which T cells and natural killer (NK) cells are absent or profoundly diminished in number, whereas B-cells are normal in number even though not functional. The discovery of the X-SCID disease gene has led to increased appreciation of the immunologic characteristics of this form of SCID and elucidation of molecular responses of lymphocytes to several cytokines. Additional molecules, involved in the intracellular communication network, have been identified as responsible of peculiar forms of SCID, including IL-7R and Janus kinase (JAK) 3 [3,4]. In most cases, these molecules are exclusively expressed in hematopoietic cells, being involved in cell development and/or functionality of terminal differentiated cells of immune system. The abundance of γc is much higher than the aliquot expressed in hematopoietic cells, thus leading to hypothesize a pleiotropic effect of the molecule [5,6].

It is noteworthy that immune and endocrine systems participate to an integrated network of soluble mediators that communicate and coordinate responsive cells to achieve effector functions in an appropriate fashion [7]. There is evidence documenting that immune cells express growth hormone receptor (GH-R) [8]. It has also recently been shown a novel dependence of GH signaling on the common cytokines receptor γc in certain cell types, suggesting an interplay between endocrine and immune systems [8]. GH-R can promote cell cycle progression of lymphoid cells and of a wide variety of other cells. Indeed, recently, it has been documented a direct involvement of γc in self-sufficient growth and GH induced proliferation in a concentration dependent manner of the molecule [9]. GH-R signaling apparatus also involves potent mitogenic molecules such as signal transducers and activators of transcription (STATs) that play a role in cell proliferation [10].

This review will focus on the relationship between different receptors that share common transducing elements and on the potential clinical implications of such still poorly understood interactions.

* Corresponding author. Fax: +39 081 545 1278.
E-mail address: pignata@unina.it (C. Pignata).

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2. γ Chain: a shared component of several cytokine receptors

The cytokines are soluble elements that control the immune and the hematopoietic system [11]. In particular, cytokines and growth factors transmit signals through specific cell-surface receptors to the nucleus by activating intracytoplasmic signaling molecules that ultimately result in the activation of transcription factors. Their functions are due to the various receptors expressed on multiple target cells [12] and their rule is closely dependent on the recognized targets.

The cytokine receptors are classified into five families on the bases of extra- and intra-cellular domains structure affinity: the cytokine receptor superfamily, interferon receptor family, tumor necrosis factor (TNF) receptor family, tumor growth factor (TGF)-β receptor family and IL-8 receptor family [13]. The cytokine receptor superfamily is the largest family, in which the receptors for IL-6, IL-11, oncostatin M (OSM), ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) contain the common gp130 [14,15], the receptors for IL-3, IL-5 and granulocyte–macrophage colony-stimulating factor (GM-CSF) share the common beta subunit [16], whereas the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 share the γc element [13].

The characterization of cytokine-activated genes, including genes regulated by γc-dependent cytokines, has long been an area of considerable interest, leading to define a prominent role for various immunological functions. An unanimously accepted series of evidence indicates that the γc-dependent cytokines control the immune response at different as well as overlapping checkpoints [17]. Most of the information so far available on the role of γc came out from studies on X-SCID in humans and in mice carrying mutations in the γc gene [1,11,18].

IL-2 is a growth factor, regulating the proliferation and apoptosis of activated T cells [19]. Moreover, IL-2 promotes NK cell cytolytic activity and immunoglobulin production by B cells [20]. IL-4 is required for the development and function of T helper 2 (Th2) cells and has an important role in allergy and immunoglobulin class switching [21]. Indeed, a role for IL-4 in B-cell Ig class-switch to IgG1 and IgE has been described [22]. IL-7 regulates lymphocyte development and homeostasis and exerts effects on both T- and B-cell biology [23–25]. In addition, IL-7 is well known for its potent role as a lymphocyte survival factor [26,27]. IL-9 is produced by a subset of activated $CD4^+$ T cells [28] and induces the activation of epithelial cells, B cells, eosinophils and mast cells [28], but its role in T cell biology remains unclear. IL-9 deficient mice have also been generated and, in these animals, the lymphoid compartment develops normally. However, these mice exhibit excessive mucus production and mast cell proliferation [29]. Interestingly, IL-9 transgenic mice develop thymic lymphomas, consistently with the presence of IL-9R in the thymus and with the ability of thymocytes to respond to IL-9 [30]. IL-15 is essential for the development of NK cells, in that mice deficient in IL-15 lack NK cells [31,32]. Furthermore, IL-15 is essential for the homeostatic proliferation of memory $CD8^+$ T cells [27]. IL-21 is the most recently described member of the γc family [33] and it has broad actions that include promoting the terminal differentiation of B cells to plasma cells, cooperating with IL-7 or IL-15 to drive the expansion of $CD8^+$ T cell populations and acting as a pro-apoptotic factor for NK cells and incompletely activated B cells [33].

In addition, γc-dependent cytokines also play an important role in supporting cell survival of activated immune cells. Clearly, regulation of cell survival and cell apoptosis is a delicate teamwork and a balanced action of all γc-dependent cytokines is of central importance. Thus, an abnormality of either one of them can have a profound impact on the homeostasis of the immune system.

Overall, in spite of the diversity of the numerous functions related to γc-containing receptors, the most important biologic effect shared between these cytokines receptors seems to be the mitogenic effect [34].

3. Signal transduction through GH receptor

GH participates to an integrated network with other mitogenic factors, as hepatocyte growth factor in liver cells, basic fibroblast growth factor in cartilage, epidermal growth factor in kidney, estrogen receptors in the uterus, bone morphogenetic proteins in various tissues, all of them being involved in tissue growth. GH is an important regulator of somatic growth, cellular metabolism, fertility and immune function. The several functions are mediated by an array of distinct signals triggered by an individual receptor, thus implying that diverse signaling pathways may be activated separately and in the context of a function specific coordinating network [35]. The GH-R was the first member of the cytokine receptor superfamily to be cloned [36]. Similarly to other members of the cytokine receptor superfamily, it consists of a transmembrane protein that contains two motifs and an extracellular domain [37]. Like other members of the family, GH-R lacks intrinsic kinase activity and signal transduction is mediated by receptor associated cytoplasmic tyrosine kinases.

A prominent role is played by the JAK2 that associates to the GH-R cytoplasmic domain [38]. After phosphorylation of JAK2, the receptor itself and several intracytoplasmic molecules are promptly phosphorylated on tyrosine residues. Further signaling proteins recruited to JAK2/GH-R complex and/or activated in response to GH include: Shc proteins that presumably lead to the activation of Ras/mitogen-activated protein kinase (MAPK) pathway [39]; insulin receptor substrates that has been implicated in the activation of phosphatidylinositol-3-kinase (PI3K) and the kinase AKT/protein kinase (PK) B [40,41]; phospholipases that lead to formation of diacylglycerol and activation of PKC and a variety of proteins that are involved in the regulation of the cytoskeleton, including focal adhesion kinase, paxillin, tensin, CrkII, c-Src, c-Fyn, c-Cbl and Nck [42,43]. This process ultimately results in the activation of STAT family members. STATs proteins dimerize and translocate into the nucleus, where they bind to specific DNA responsive elements of GH target genes, eventually inducing the activation of gene transcript [44].

The duration of GH-activated signals is a key factor in relationship to the biological actions of the hormone. Removal of cell surface GH-R by endocytosis is an early step in the termination of GH-dependent signalling [45]. Furthermore, suppressors of cytokine signaling (SOCS) proteins act as negative regulators of the main cytokine-activated signaling pathway, the JAK/STAT signal cascade [46]. Moreover, several studies documented that there are at least three different phosphatases involved in the specific down-regulation of GH-R signaling: SH2 domain-containing protein-tyrosine phosphatase (SHP)-1; tyrosine-protein phosphatase (PTP)-1B and PTP-H1 [47].

This knowledge would help understand tissue specificity of GH action and would allow devise strategies to enhance individual functions of GH. Thus, pharmacological targeting of specific negative regulators of GH signaling would have a remarkable potential to enhance the beneficial effects of GH [46].

4. JAK/STAT signaling

Following the interaction of cytokines and growth factors with their receptors [48,49], the tyrosine kinases bind and phosphorylate the cytoplasmic tail of the receptors [50]. In this process, the JAK family members play a prominent role. Thus far, four distinct members of the JAK family are known in humans: JAK1, JAK2, JAK3 and Tyk2.

Following receptor dimerization, JAKs activate downstream molecules through three different transduction mechanisms. First, the phosphorylated cytokine receptor may associate with the adaptor Shc, which is itself phosphorylated and binds to Grb2 [51]. Grb2 may thus anchor to the Ras guanine nucleotide exchanging factor (Sos) [52]. Membrane translocation of the Grb2/Sos complex catalyzes the conversion of inactive GDP-bound Ras to the active GTP-bound state [53]. This results in the activation of Raf-1, MAPK and eventually in the induction of immediate-early genes (c-fos and c-jun) [54]. Second, JAKs may bind and phosphorylate insulin receptor substrates (IRSs). Indeed, JAK activation by interleukins results in the phosphorylation of IRS-1 [55]. Once activated, IRS may bind the PI3K. In addition to promoting PI3K activation, tyrosine phosphorylated IRS may recruit Grb2 and thus amplify the Ras/Raf-1 signaling pathway [56].

A third essential component of the JAK signaling pathway is the phosphorylation of the class of transcription factors known as STAT molecules [57]. The STATs factors comprise a diverse group of cytoplasmic proteins that are involved in several functions, such as regulation of the expression of effector genes, cell differentiation, survival and apoptosis [47]. So far, seven mammalian STATs: STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 have been characterized [47]. The STATs contain a tyrosine residue that may undergo JAK-mediated phosphorylation, and they also contain SRC homology (SH) 2 and SH3 domains. Following triggering of the JAK-mediated signaling pathway, STATs may interact with the cytokine receptor complex by binding via their SH2 domain to the phosphotyrosine of the cytokine receptor chain [57]. Following dimerization, STATs translocate to the nucleus, where they bind to consensus sequences in the enhancer elements of the promoter regions of target genes and favour gene transcription. Gene accessibility to STAT binding is another mechanism through which specific responses to distinct cytokines are obtained. It has been suggested that JAK-dependent STAT activation is more crucial to cell differentiation than to proliferation. The specificity of the response to cytokines is largely dependent on the particular combination of STATs recruited by the different signal-transducing chains of the cytokine receptor.

STAT proteins are essential regulators of cell proliferation, differentiation and survival in different cellular contexts, thus revealing their critical role in malignant transformation. STATs molecules have been demonstrated to directly participate in tumor development and progression [58]. Knockout studies have also highlighted the function of STAT proteins in the development and function of the immune system and of their roles in maintaining peripheral immune tolerance and tumour surveillance.

STATs are activated by a number of cytokines, including interferons and interleukins, as well as growth factors and hormones. STAT1 is inducible by interferon (IFN)-α/β and IFN-γ and is involved in anti-viral and anti-bacterial response, in growth inhibition, apoptosis and tumor suppression [47]. STAT3 is mainly activated by IL-6 and epidermal growth factor (EGF) and is involved in mitogenesis, survival, anti-apoptosis and oncogenesis [59]. STAT4 is predominantly stimulated by IL-12 and is involved in Th1 development in humans. This molecule is also activated by IL-23 in murine cells and, additionally, by IFN-α in human cells, being recruited to type I IFN receptor through interaction with STAT2 [47]. STAT6 molecule is activated by IL-4 and participates in Th2 development [58]. STAT5a and STAT5b are involved in prolactin and growth hormone signaling. STAT3 and STAT5, have been demonstrated to directly participate in tumor development and progression [60,61].

STATs participate in oncogenesis through up-regulation of genes encoding apoptosis inhibitors and cell cycle regulators such as Bcl-x_L, Mcl-1, cyclins D1/D2, and c-Myc [62–64]. Moreover, tumor cells possessing activated STAT3 or STAT5 are predicted to be resistant to chemotherapeutic agents that may utilize similar apoptotic pathways. It has been clearly documented that inhibition of constitutively active STATs results in growth inhibition and induction of apoptosis in tumor cells [61,65].

Recent studies have shown that JAK/STAT signaling can be regulated through distinct mechanisms. Down-regulation of cytokine-JAK/STAT signaling is important for homeostasis and the prevention of chronic inflammation or autoimmunity. Moreover, also constitutive inhibitory pathways and inducible mechanisms have been described. Constitutive inhibitory mechanisms include the proteolysis, dephosphorylation and interaction with inhibitory molecules termed protein inhibitors of activated STATs (PIAS) [66]. Regulated or inducible inhibitory mechanisms have been identified. The receptor expression is down-regulated, through the induction of inhibitory molecules termed SOCS proteins and by rapid MAPK or PKC-dependent modification of pre-existing signaling components.

A potential novel pharmacological strategy may be to develop specific drugs that can specifically target the JAK-STAT regulators or the motifs implicated in such intermolecular interactions.

5. Networking between γc and GH-R signaling: atypical patients as "nature experiments"

A potential role of γc in GH-R signaling has been proposed on the basis of the impairment of various GH-induced events in γc deficient conditions. First, the signal transduction properties of GH-R in B-cell lines from X-SCID patients following GH stimulation is abnormal, in that GH stimulation fails to induce phosphorylation on tyrosine residues of several proteins, including STAT5 molecule [8].

Recent evidence indicates that silencing of γc induces a considerable decrease of the protein amount in lymphoblastoid cell lines that results in a reduction of self-sufficient growth in a concentration dependent manner along with a decrease of the response of lymphoblastoid cells to GH-induced proliferation and STAT5 subcellular redistribution following GH-R perturbation [9]. In addition, the activation of JAK3 is a downstream event of γc activation and a correlation between γc amount and the extent of constitutive activation of JAK3 has been documented [9]. Taken together, these data imply a direct involvement of γc in the control of cell cycle progression. Previously, it has been reported on a patient affected with X-SCID, short stature and peripheral GH hyporesponsiveness, an abnormal protein phosphorylation that normally occur following GH-R stimulation [67]. Of note, in this patient the immunological reconstitution through bone marrow transplantation paralleled the restoring of GH-R functionality, which resulted in a normal production of insulin growth factor I (IGF-I) [68].

This would also imply that haematopoietic-derived cells represent an important source of those intermediate molecules that play a role in the GH-R functionality.

6. Clinical implications of alterations of GH-R/IGF-I axis in immune response and abnormal cell growth

The effects of GH on growth are mostly mediated by intermediate factors [69]. GH upon binding to its receptor initiates the signaling cascade, which culminates in the regulation of multiple genes, including IGF-I and its major binding protein, the IGF binding protein-3 (IGFBP-3). IGF-I with the IGFBP-3 and the acid labile subunit (ALS) is released into the circulation as a ternary complex [70].

Evidence supports a role for GH acting as a cytokine in the immune system under conditions of stress, counteracting immunosuppression by glucocorticoids [71]. Lymphoid cells express the

GH-R and GH can be produced by immune tissues, suggesting an autocrine/paracrine mode of action of GH. Moreover, GH can, directly or indirectly through the production of IGF-I, promote cell cycle progression and prevent apoptosis of lymphoid cells and of a wide variety of other cells, as well. It has been demonstrated that both GH and IGF-I are able to promote cell survival and proliferation through independent different pathways, thus indicating a potential function related specificity of the individual pathway [72]. It has been suggested that GH treatment may partially protect immune cells against apoptosis induced by stress conditions and deregulated expression of GH may participate to the development of malignancies of immune cells, such as leukemias or lymphomas [71]. Moreover, IGF-I induces a number of biologic effects, as induction of cell growth through the activation of cell cycle machinery, maintenance of cell survival by acting on the Bcl family members and induction of cellular differentiation through still poorly characterized mechanisms [73]. Overall, IGF-I inhibits apoptosis as well, thus acting as cell survival factor [74]. Components of the IGF-I system may play a key role in the deregulation of cell cycling or apoptosis in tumor growth [75].

As for the relationship between the GH/IGF-I axis and the risk of developing cancer, no conclusive data are available. There is evidence indicating that the GH/IGF-I axis has a role in the development of cancer through the regulation of cell proliferation, differentiation and apoptosis [76]. In particular, the association between circulating IGF-I and IGFBP-3 concentrations and the risk of developing cancer was documented [77]. IGF-I is mitogenic *per se* and exerts an important antiapoptotic effect, whereas IGFBP-3, which is thought to inhibit growth through ligand sequestration, is supposed to also have antiproliferative and proapoptotic effects, thus interfering with tumor growth [77]. Moreover, in the transformed cell, there are several data showing that IGF-I-R regulates cancer cell proliferation, survival and metastasis [78]. Differently from IGF-I and IGFBP3, the involvement of GH in the physiopathology of cancer is an open issue.

Progress in defining the pathogenic implications of IGF-I/IGF-I-R and downstream molecules in neoplasia might lead to the development of novel targeting strategies to fight those cancers that may be proven responsive. Therefore careful attention to future clinical applications of these therapeutic targeting in combination with chemotherapy will be necessary [79].

The GH-R signaling apparatus also involves potent mitogenic molecules such as γc and STATs that play a role in the cell proliferation and, in general, in cell homeostasis. It should be noted that overexpression of γc, in patients treated with gene therapy for X-SCID, resulted in lymphoproliferation, as a consequence of insertional oncogenesis in LMO2 oncogene [80,81]. However, the insertional mutagenesis was not found in all patients who developed the lymphoproliferative disorder, thus suggesting a direct involvement of γc in self-sufficient growth and activation induced proliferation [9].

Clinical studies have greatly contributed in defining that STATs are key molecules in GH-R signaling and in understanding the mechanisms by which GH activates genes that lead to its physiological functions. In particular, STAT5b appears to be involved in GH mediated IGF-I gene transcription and production of IGF-I and in transcription and production of IGFBP-3 and the ALS as well [82]. In patients carrying mutations of STAT5b gene a marked reduction of the GH-dependent peptides IGF-I, IGFBP-3 and ALS has been observed [83–85] while basal and stimulated GH concentrations were either normal or increased. These patients were characterized by growth failure and immunodeficiency [83–87].

However, the relationship between endocrine and immune dysfunctions in patients with STAT5b alterations are not yet completely defined [88]. Certainly, STAT5b seems a shared component between signaling pathways implicated in both immunological and endocrine functions. Even though, any cytokines, as IL-2, IL-7, IL-21 and IFN-γ, can activate STAT5b. Most of the current knowledge about the biological function of STAT family members has been achieved through disruption approaches and studies of knock-out (KO) mice [48]. In particular, it has been supposed that STAT5a/b would have very fundamental functions in regulating cell growth. Indeed, STAT5a and STAT5b KO mice have a most important failure of several responses associated with growth hormone secretion. In particular, the deletion of STAT5b alone gives a phenotype analogous to that observed in GH-R deficient mice, resulting in a failure of postnatal growth.

The role of STAT5 in the immune system has also been extensively analyzed. Splenocytes from STAT5a KO mice have a partial defect in anti-CD3-induced proliferation that can be overcome by high doses of IL-2 [89]. Moreover, also splenocytes from STAT5b KO mice exhibit greatly diminished proliferation in response to IL-2 and IL-15. Moreover, STAT5b is essential for potent NK cell-mediated proliferation and cytolytic activity [90]. However, an impaired proliferation of peripheral T lymphocytes has been observed in STAT 5a/b KO mice, even if this feature is, probably, due to a defect in the cell cycle entry rather than to a decreasing of IL-2 receptor expression. In fact, while lymphopoiesis is normal, T cells from double KO mice show a marked failure to undergo cell cycle progression and a diminished expression of proteins fundamental for proliferation [91].

Thus, it is clear that STAT5 proteins are strongly correlated with some oncogenic events, such as proliferation and apoptosis [91]; so, the therapeutic inhibition of these transcription factors may be proven helpful for those diseases characterized by an alteration of cell growth homeostasis. Moreover, the development of selective inhibitors of STAT activation may be a promising area in the field of novel anticancer therapeutics [61].

It remains to be further elucidated whether the γc involvement is required for the expression of the biologic effects of GH and its intermediate molecules on cell growth in either physiological or pathogenic conditions.

Conflict of interest

None.

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Networking Between γc and GH-R Signaling in the Control of Cell Growth

Rosa Romano[1], Loredana Palamaro[1], Giancarlo Parenti[1], Mariacarolina Salerno[1], Anna Fusco[1], Pietro Vajro[2], Donatella Capalbo[1], Brigida Ranieri[1], Roberta Naddei[1] and Claudio Pignata[1,*]

[1]*Department of Pediatrics,"Federico II" University, Naples, 80131, Italy;* [2]*Chair of Pediatrics, Medical School of Salerno, Salerno, Italy*

Abstract: The family of type I cytokines, including IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21, shares common transducing element the common cytokine receptor γc. The receptors containing γc exert prominent mitogenic effects and play an important role in several immunological functions and in supporting cell survival. The γc-dependent cytokine receptors use the Janus Kinase (JAK)/Signal transducer and activator of transcription (STAT) signaling pathway to mediate gene activation or repression. The Growth Hormone (GH) is a peptide of 191 amino acids and 22 kDa molecular weight, produced by the adenohypophysis, that regulates many important functions, as control of cellular metabolism, immune functions, fertility and somatic growth. Of note, the existence of a previously unappreciated functional interaction between γc and Growth Hormone receptor (GH-R) has been recently documented. The impairment of various GH-induced events in patients affected with severe combined immunodeficiencies due to γc defects suggests a potential functional interaction between GH-R pathways and γc, indicating a further link between endocrine and immune system with potential implication of the molecule in the cell cycle progression and control. GH-R pathways and γc interaction leads to the activation and intranuclear translocation of STAT5b protein. Moreover, evidence suggests that both GH-induced and spontaneous cell cycle progression and cell growth are strongly dependent on the amount of γc expression. To date, the regulation of cell survival and apoptosis can be considered a delicate teamwork and a proper functionality of γc-dependent cytokines on the whole seems to play prominent roles, as revealed by the profound impact that their abnormal function can have on the homeostasis of the immune system.

Keywords: Cell growth, γc, GH, GH-R, JAKs, STATs.

INTRODUCTION

The cytokines and growth factors are important regulator elements. They transduce signals to the nucleus though specific cell-surface receptors and the process ultimately results in the activation of transcription factors [1]. Cytokines can be considered structurally distinct ligands binding different classes of receptors [2]. The cytokine receptors could be classified on the bases of capability to recognize different ligands through their extra- and intra-cellular domains structure: cytokine receptor class 1 superfamily, interferon receptor family, tumor necrosis factor (TNF) receptor family, tumor growth factor (TGF)-β receptor family and IL-8 receptor family [3]. These cytokines exert an important role in immunology, in that they are involved in initiating innate immunity, orchestrating adaptive immune mechanisms and constraining immune and inflammatory responses [2].

The common cytokine receptor γ chain (γc) was first discovered as a component of the IL-2 receptor, which is the prototypical member of the cytokine receptors sharing γc. *IL2RG* gene encoding γc is localized to chromosome Xq13 [4,5]. Mutations in this gene are responsible for the X-linked Severe Combined Immunodeficiency (X-SCID). SCIDs represent a wide spectrum of illnesses, which differ in either the qualitative or quantitative alterations of T-, B- and Natural Killer- (NK-) cells [6-11] and the X-SCID represents the most common form of SCID, accounting for 40–50% of all cases SCID. In particular, this form of SCID is characterized by the complete absence of both T and NK lymphocytes, whereas B cell number is normal [5].

The receptors containing γc element exert mitogenic effects and regulate lymphocyte development and function [12]. To date, the regulation of cell survival and apoptosis can be considered a delicate teamwork and a proper functionality of γc-dependent cytokines on the whole seems to play prominent roles, as revealed by the profound impact that their abnormal function can have on the homeostasis of the immune system [13].

Of note, the existence of a previously unappreciated functional interaction between γc and GH-R has been documented. This interaction leads to the activation and intranuclear translocation of STAT5b protein [14]. This relationship was suggested by an initial serendipitous observation on a patient affected with X-SCID, who showed during the follow up a severe impairment of body growth and after the exclusion of a GH deficiency [15,16] was suspected to have an idiopathic short stature and peripheral insensitivity to GH [17,18], similarly to what happens in the presence of GH-R mutation [19]. In this patient, mutational analysis of a few candidate genes, functionally related to short stature and potentially to the immune defect, as GH-R

*Address correspondence to this author at the Department of Pediatrics, "Federico II" University, Naples, via S. Pansini 5 - 80131, Italy; Tel: + 39 081 7464340; Fax: + 39 081 5451278; E-mail: pignata@unina.it

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itself, JAK2 and STAT5 failed to reveal any further alteration that could explain the GH-R unresponsiveness [17].

This review focuses on the γc as a common transducing element shared between several cytokines and growth hormone receptors, indicating a further functional link between endocrine and immune system with potential implication of the molecule in the cell cycle progression and control.

THE RECEPTORS SHARING γC ELEMENT AND INTRACELLULAR TRANSDUCTION SIGNALING

One important family of type I cytokines is the common cytokine receptor γc family, including IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 [3], which plays important roles in immunological functions and in supporting cell survival (Table **1**).

IL-2 is a glycoprotein secreted by activated T lymphocytes, which, in turn, stimulates the development of regulatory T (T_{Reg}) cells and peripheral T cell tolerance [20], as well as the proliferation and apoptosis of activated T cells, with an autocrine effect [21]. Moreover, it promotes the increase of NK cell cytolytic activity and immunoglobulin production by B cells [22].

IL-4 is secreted by T lymphocytes and is required for the development and function of T helper 2 (Th2) cells. In addition, IL-4 also plays a pivotal role in allergy and immunoglobulin class switching. Indeed, a role for IL-4 in B cell Ig class-switch to IgG1 and IgE has been described [23].

IL-7 regulates survival, development and homeostasis of T lymphocytes, both in humans and mice [24,25]. Indeed, alterations of the IL-7-induced signaling apparatus profoundly affects T cell development, as observed in patients with X-SCID [26], as well as in patients with SCID due to mutations in Janus kinase 3 (*JAK3*) [27], or by mutations in *IL7RA* [28]. Moreover, IL-7 is also required for the development of B cells only in mice [29].

Activated $CD4^+$ T cell population is able to produce IL-9, which induces the activation of epithelial cells, B cells, eosinophils and mast cells [30], even though the molecular mechanism activated by this cytockine in T cell remains to be elucidated.

IL-15 induces differentiation of NK cells and is essential for their functionality [31,32]. Defects in IL-15-mediated signaling result in the failure of NK cell development, as reported in X-SCID and JAK3-deficient SCID patients [29]. Furthermore, IL-15 is essential for the homeostatic proliferation of memory $CD8^+$ T cells [25].

Recently a new member of γc sharing cytokine receptors, the IL-21R has been identified [33]. IL-21 has broad actions that include promotion of the terminal differentiation of B cells to plasma cells, cooperation with IL-7 or IL-15 to drive the expansion of $CD8^+$ T cell populations and an action as a pro-apoptotic factor for NK cells, as well as for incompletely activated B cells [25,33]. In addition, it has been reported that the IL-21 promotes the development of type 1 diabetes mellitus [33] and systemic lupus erythematosus (SLE) in animal models [34].

The γc-dependent cytokine receptors use the Janus Kinase (JAK)/Signal transducer and activator of transcription (STAT) signaling pathway to mediate gene activation or repression [35] (Fig. (**1**)). The JAK/STAT pathway is one of pleiotropic cascades used by mammalian cells to transduce intracellular signals necessary to the development and homeostasis [36]. The binding of the ligand to the specific receptor and the subsequent receptor subunits dimerization leads to the recruitment of JAKs kinases into close proximity of receptors, allowing their trans-phosphorylation and activation. The activated JAKs initiate the intracellular signal transduction through the phosphorylation of tyrosine motifs present in the receptor cytoplasmic domains and in receptor-associated proteins. Four distinct members of JAK family are known in humans: JAK1, JAK2, JAK3 and Tyk2, while seven STAT proteins (STATs) have been identified, named STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6 [37]. STATs contain a tyrosine residue and also SH2 and SH3 domains that undergo JAK-mediated phosphorylation [38].

All γc-dependent cytokine receptors are able to activate JAK1 and JAK3 proteins, particularly JAK3, which selectively associates with γc [39] and can phosphorylate only STAT3 and STAT5 molecules. Differently, JAK1 can

Table 1. The Biological Roles of Cytokine Receptors Sharing the γc Element

Cytokine	Biological Roles
IL-2	Stimulates the development of T_{Reg} cells, peripheral T cell tolerance, proliferation and apoptosis of activated T cells. Increases NK cell cytolytic activity and immunoglobulin production by B cells
IL-4	Stimulates the development and function of Th2 cells and is implicated in immunoglobulin class switching
IL-7	Regulates survival, development and homeostasis of T lymphocytes both in humans and mice
IL-9	Induces the activation of epithelial cells, B cells, eosinophils and mast cells
IL-15	Regulates differentiation and functionality of NK cells and is essential for the homeostatic proliferation of memory $CD8^+$ T cells
IL-21	Promotes terminal differentiation of B cells to plasma cells and, by cooperating with IL-15, drives the expansion of $CD8^+$ T cell populations. Acts as a pro-apoptotic factor for NK cells

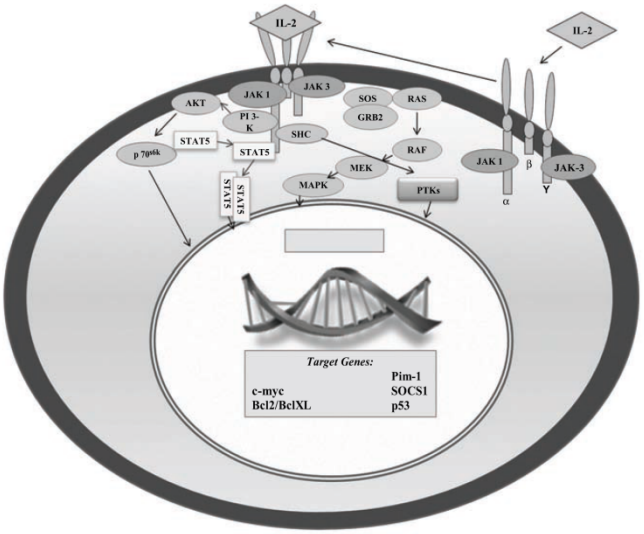

Fig. (1). Intracellular transduction signaling apparatus involving the γc molecule. The γc-dependent cytokine receptors use JAKs kinase signal transducer and STAT proteins to activate the transcription of selected genes that exert mitogenic effects and regulate lymphocyte development, function and survival, such as cyclins, Bcl2/BclXL, p53.

phosphorylate STAT1, STAT3 and STAT5, but only in the presence of an activated JAK3 [40]. Phosphotyrosine-containing motifs in receptor cytoplasmic domains act as docking sites for many signaling proteins, including STATs.

Several molecules are able to activate STAT proteins, including interferons, interleukins and growth factors and hormones. In particular, interferons (IFN)-α/β and IFN-γ lead to a STAT1-mediated anti-viral and anti-bacterial response, growth inhibition, apoptosis and stimulation of tumor suppression [37]. STAT3 is mainly activated by IL-6 and epidermal growth factor (EGF) and is involved in mitogenesis, survival, anti-apoptosis and oncogenesis [41]. STAT4 is predominantly stimulated by IL-12 and is involved in Th1 development in humans. This molecule is also activated by IL-23 in murine cells and, additionally, by IFN-α in human cells, being recruited to type I IFN receptor through interaction with STAT2 [37]. STAT6 molecule is activated by IL-4 and participates in Th2 development. STAT5a and STAT5b are involved in prolactin and growth hormone signaling [42]. STAT3 and STAT5 have been demonstrated to directly participate in tumor development and progression [43,44].

STATs are activated by phosphorylation of conserved tyrosine residue. This event leads to the dissociation of STATs from the receptor, dimerization and acquisition of a high-affinity DNA-binding activity. After the activation, STATs translocate to the nucleus, where they bind to gene promoters and activate transcription of genes involved in cell proliferation, differentiation and survival [36,37,45]. In addition, has been reported the role of STATs molecules in tumor development and progression [46].

Of note, STATs play important roles in oncogenesis by up-regulation of genes encoding apoptosis inhibitors and cell cycle regulators, namely oncogenes, such as Bcl-xL, Mcl-1, cyclins D1/D2, and c-Myc [47]. It has been also documented a constitutive activation of STAT3 or STAT5 in tumor cells resistant to chemotherapeutic agents, that exert their effect on cell apoptosis machinery [43].

Recently, it has also been shown that the inhibition of cytokine-JAK/STAT signaling may be useful for homeostasis and the prevention of chronic inflammation or autoimmunity [48,49]. Proteolysis, dephosphorylation and interaction with inhibitory molecules termed protein inhibitors of activated STATs (PIAS) are responsible for the constitutive inhibitory mechanisms of STATs [50]. Suppressors Of Cytokine Signaling (SOCS) proteins are inhibitory molecules, which down-regulate the receptor expression, by rapid MAPK or PKC-dependent modification [51].

The development of drugs specifically targeting the JAK-STAT regulators or the motifs implicated in such intermolecular interactions might be a potential novel pharmacological therapeutic approach in the therapy of neoplastic disorders.

Recently, we documented that γc is implicated in the control of cell proliferation of malignant hematopoietic cells [52]. Moreover, using of knock-out strategy or through the neutralization of cytokines receptors involving γc in the

signaling apparatus, an anticancer effect has been recently documented [53,54]. This evidence is in keeping with a role of γc as a cofactor in tumor growth. Future research strategies might be designed to evaluate γc levels in different primary leukemic cell lines, in order to compare the amount of the molecule to the type of the tumor or to its aggressiveness. Future studies might clarify whether the levels of protein expression may be used as prognostic factor.

BIOCHEMICAL PATHWAYS INVOLVED IN GROWTH HORMONE RECEPTOR SIGNALING

The GH is a peptide of 191 amino acids and 22 kDa molecular weight, produced by the adenohypophysis, that regulates many important functions such as control of cellular metabolism, immune functions, fertility and somatic growth [55-57]. GH and other mitogenic factors, including hepatocyte growth factor in liver cells, basic fibroblast growth factor in cartilage, epidermal growth factor in kidney, estrogen receptors in the uterus, bone morphogenetic proteins in various tissues, participate to a wide network of well integrated signals [55]. The several functions mediated by GH are activated following GH-R stimulation, the first identified member of the cytokine receptor class 1 superfamily, which includes receptors for Erythropoietin (EPO), Granulocyte colony stimulating factor (G-CSF), Granulocyte-macrophage colony stimulating factor (GMCSF), IL-2 - 7, IL-9, IL-11, IL-12 and many other cytokines [58].

The members of the cytokine receptor superfamily 1 lack intrinsic kinase activity, thus requiring the recruitment of cytoplasmic tyrosine kinases to the intracellular signaling apparatus [59,60]. In particular, JAK2 is required for a fully functional GH-R-mediated pathway [61]. The receptor structure consists of a transmembrane protein with two motifs, an extracellular domain, which binds the ligand and an intracellular domain associated to JAK2 [59].

After the phosphorylation of JAK2, the receptor itself and several intracytoplasmic molecules are promptly phosphorylated on tyrosine residues. Further signaling proteins recruited to JAK2/GH-R complex and/or activated in response to GH include: Shc proteins, that presumably lead to the activation of Ras/mitogen-activated protein kinase (MAPK) pathway [62], insulin receptor substrates, that have been implicated in the activation of phosphatidylinositol-3-kinase (PI3K) and the kinase AKT/protein kinase (PK) B [63], phospholipases, that lead to the formation of diacylglycerol and subsequent activation of PKC and a variety of proteins involved in the regulation of the cytoskeleton, including focal adhesion kinase, paxillin, tensin, CrkII, c-Src, c-Fyn, c-Cbl and Nck [64-66].

These observations imply that GH-R, as well as other receptors, is able to integrate different pathways differentially regulated. Several causes of one of these pathways may cause features of altered GHR signaling [67]. It has been shown that different cell types as hepatocytes, fibroblasts and myoblasts have different regulatory mechanisms which lead to cell specific functions of GH [68]. In keeping with this, it has been observed a cell type-restricted STATs activation [69]. STAT5 is not activated following GH stimulation in human fibrosarcoma cells even though these cells express the STAT5 protein [70], thus implying that a selectivity in the involvement of specific STAT subset seems to be a general feature of GH-R signal transduction.

Moreover, it has been documented that GH and IGF-I are able to activate several pathways involved in the control of cell survival and proliferation of lymphoid cells, directly or indirectly through the production of IGF-I [69]. Of note, IGF-I activates a number of biologic effects, as induction of cell growth, maintenance of cell survival by acting on the Bcl family members and induction of cellular differentiation through still poorly characterized mechanisms [71]. Overall, IGF-I inhibits apoptosis as well, thus acting as cell survival factor [70]. Components of the IGF-I system may play a key role in the deregulation of cell cycling or apoptosis in tumor growth [72]. In keeping with this observation, it has been documented that the treatment with GH may increase the risk of developing hematopoietic malignancies, including leukemias or lymphoma [68]. Moreover, evidence suggests a potential role for the GH/IGF-I axis in the development of cancer through the regulation of cell proliferation, differentiation and apoptosis [73]. In addition, it has been documented a positive correlation between circulating IGF-I and IGFBP-3 levels and the risk of developing cancer [74]. Particularly, IGF-I is mitogenic *per se* and exerts an important antiapoptotic effect, whereas IGFBP-3, which is thought to inhibit growth through ligand sequestration, is also supposed to have antiproliferative and proapoptotic effects, thus interfering with tumor growth [74]. Nevertheless, no conclusive data are available of the relationship between the GH/IGF-I axis and the risk of developing cancer.

As matter of fact, progress in defining the pathogenic implications of GH/IGF-I/IGF-I-R and downstream molecules in neoplasia might lead to the development of novel targeting strategies to fight those cancers that may be proven responsive.

THE LINK BETWEEN γC AND GH-R PATHWAYS: THE CONTROL OF CELL GROWTH

The impairment of various GH-induced events in γc deficiencies suggests a potential interaction between GH-R pathways and γc [14]. Particularly, an altered STAT5 phosphorylation following GH-R stimulation in a patient affected with X-SCID and peripheral GH hyporesponsiveness with short stature and delayed bone age, has been described [17]. Allogeneic bone marrow transplantation not only brought along a fully competent immunological reconstitution, but also caused a considerable improvement in linear growth and restored basal and GH-induced levels of insulin growth factor I (IGF-I) [75]. Afterwards, it has been reported an abnormal signal transduction of GH-R in B cell lines (BCLs) from X-SCID patients following GH stimulation. GH-R activation fails to induce phosphorylation on tyrosine residues of STAT5 and its nuclear translocation, thus no functional effect is observed in these cells [14]. Of note, *IL2RG* gene transduction of X-SCID cells with WT γc gene, restored GH induced proliferation and STAT5 nuclear translocation [14].

Finally, a direct role of γc in the regulation of cell cycle progression and in the control of both GH-induced cell and spontaneous growth has been proposed on the basis of γc silencing experiments. Effects of GH stimulation in γc-

silenced or X-SCID BCLs have been compared with control BCLs. In γc-silenced or X-SCID BCLs, recombinant GH induced proliferation at a much lower extent, while no change in subcellular redistribution of STAT5 has been observed. In control BCLs, GH stimulation determines a rapid increase of nuclear amount of STAT5 and enhances proliferation. Thus, the γc-silencing results in the severe decline of self-sufficient growth in BCLs, as shown by comparing the ability to proliferate of γc-silenced BCLs and control cells [76]. In addition, the activation of JAK3 is a downstream event of γc activation and a correlation between γc amount and the extent of constitutive activation of JAK3 has been associated to autonomous cell growth and malignant transformation of lymphoid cells [76]. Of note, a relationship between γc expression and the amount of constitutively activated JAK3 has been shown. In particular, a higher constitutive activation of JAK3 was found in control BCLs, whereas a decrease in phospho-JAK3 levels was observed in γc-silenced and in X-SCID BCLs, despite a comparable amount of the whole protein. Moreover, the amount of constitutive JAK3 paralleled the amount of γc [76]. These data suggest that both GH-induced and spontaneous cell cycle progression and cell growth are strongly dependent on γc expression.

Recently, clinical gene-therapy trials using ex-vivo retroviral vectors, have been proven as a corrective therapeutic approach for X-SCID in humans [77]. In particular, immunological reconstitution has been documented in 17 out of 20 patients enrolled in two distinct clinical trials through gene therapy approach [77]. Unfortunately, 5 of these patients developed a lymphoproliferative disorder [78]. Even though it has been reported that retroviral integration of the corrective IL2RG occurred near the locus of LMO2 oncogene and may have upregulated LMO2 expression [79], further studies documented that leukemogenesis was not due to insertional mutagenesis and raised the possibility that IL2RG may be oncogenic *per se* [80]. In addition, proliferation and transformation of normal hematopoietic and laeukemic cells has been observed with supra-physiological doses of GH [81]. The risk of developing cancer is determined by a combination of genetic factors and environmental effects, in particular diet and lifestyle. There is increasing evidence that the GH/IGF-I axis may provide a link between these factors. GH and related signaling molecules, through their implication in the regulation of normal cell proliferation, differentiation and apoptosis, may act as a cofactor in the overgrowth of cancer cells [73]. Therefore, the evidence that these molecules may be implicated in the control of tumor progression highlights the need to go further in a detailed understanding of the mechanisms underlying the process aimed to identifying new potential therapeutic targets.

CONCLUSIONS

Immune and endocrine systems participate to an integrated network of soluble mediators that communicate and coordinate responsive cells to achieve effector functions in an appropriate fashion [68]. Several evidences demonstrate the existence of a previously unappreciated relationship between distinct elements, such as GH-R and γc and their signaling pathways. Crosstalk between receptor signaling systems is now emerging as an important and exciting area of signaling research.

The impairment of various GH-induced events in γc deficiencies suggests a potential interaction between GH-R pathways and γc, indicating a further functional link between endocrine and immune system with potential implication of the molecule in the cell cycle progression and control. The existence of shared molecular mechanisms of transduction between immune and endocrine systems is well documented in several autoimmune disorders characterized by both endocrine and immune features [82-85]. GH-R pathways and γc interaction leads to the activation and intranuclear translocation of STAT5b protein. The signals mediated by STATs generally play a central role in the control of important cellular events such as cell proliferation, differentiation and apoptosis [46], even though the overall role of the STAT molecules in GH-R signal transduction has not been fully elucidated. The overall signal transduction properties of GH-R in X-SCID patients and control BCLs following GH stimulation, have been recently elucidated. In particular, after GH stimulation no phosphorylation of STAT5 protein was observed in γc negative patients cell lines in contrast to the control cells, in which a prompt activation of STAT5 occurred. Of note, reconstitution of X-SCID cells with WT γc gene corrected the functional and biochemical abnormalities resulting in an appropriate nuclear translocation of STAT5. These findings strongly support an essential role of γc in GH-R signaling. Of note, a co-localization of GH-R and γc has been documented. Even though a physical interaction between the two proteins may be possible, any physical association has not yet been documented [14]. Currently, studies are ongoing to identify potential physical interactions between the two proteins. Moreover, evidence suggests that both GH-induced and spontaneous cell cycle progression and cell growth are strongly dependent on γc expression. Whether the participation of γc to the GH-R confers some additional properties to the receptor in hematopoietic cell differentiation and functioning remains to be elucidated.

DISCLOSURE

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CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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CLINICAL REPORT

AMERICAN JOURNAL OF medical genetics PART A

Noonan-Like Syndrome With Loose Anagen Hair Associated With Growth Hormone Insensitivity and Atypical Neurological Manifestations

D. Capalbo,[1] D. Melis,[1] L. De Martino,[1] L. Palamaro,[1] S. Riccomagno,[2] G. Bona,[2] V. Cordeddu,[3] C. Pignata,[1] and M. Salerno[1]*

[1]Dipartimento di Pediatria, Università degli Studi di Napoli Federico II, Napoli, Italy

[2]Dipartimento di Scienze Mediche, Divisione di Pediatria, Università del Piemonte Orientale, Novara, Italy

[3]Dipartimento di Ematologia, Oncologia e Medicina Molecolare, Istituto Superiore di Sanità, Roma, Italy

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Noonan-like syndrome with loose anagen hair (NS/LAH; OMIM 607721), recently related to the invariant c.4A>G missense change in *SHOC2*, is characterized by features reminiscent of Noonan syndrome. Ectodermal involvement, short stature associated with growth hormone (GH) deficiency (GHD), and cognitive deficits are common features. We report on a patient with molecularly confirmed NS/LAH exhibiting severe short stature associated with GH insensitivity (GHI), and chronic complex tics, a neurological feature never described before in this syndrome. IGF1 generation test revealed only a blunted increase in IGF1 after exogenous GH treatment, revealing mild GH insensitivity associated with proper STAT5 activation. Most common causes of secondary tics in childhood were excluded.

Key words: chronic tics; GH insensitivity; Noonan-like syndrome with loose anagen hair; SHOC2

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INTRODUCTION

Noonan syndrome (NS; OMIM #163950) is a clinically variable disorder characterized by postnatally reduced growth, distinctive facial dysmorphism, cardiac defects and variable cognitive deficits. It is caused by aberrant signal flux through RAS and mitogen activated protein kinase (MAPK) signaling. Mutations in several genes coding for proteins involved in this pathway (*PTPN11*, *SOS1*, *KRAS*, *RAF1*, *BRAF*, and *MEK1*) have been identified [Tartaglia et al., 2011]. All these signal transducers positively contribute to RAS-MAPK signaling and possess autoinhibitory mechanisms that are impaired by mutations.

Short stature is one of the most common features observed in NS as well as in other disorders associated to RAS-MAPK dysregulation. It is well known that the RAS-MAPK transduction pathway plays a key role in growth hormone (GH) signaling [Padidela et al., 2008]. However, the exact mechanism of impaired GH-IGF1-axis in patients with aberrant RAS-MAPK signaling is still controversial. An altered response of GH to stimulation tests has been reported in some children [Cotteril et al., 1996; Romano et al., 1996], whereas other authors have reported normal GH secretion with low IGF1 levels suggesting GH insensitivity (GHI) [Ferreira et al., 2005; Limal et al., 2006].

Mazzanti et al. [2003] first described a clinically distinctive syndrome tentatively named "Noonan-like syndrome with loose anagen hair" (NS/ LAH OMIM60772) characterized by features reminiscent of NS and a unique pattern of ectodermal abnormalities. Recently, Cordeddu et al. discovered that an invariant mutation in *SHOC2*, c.4A>G, predicting the p.Ser2Gly change in the encoded protein, underlies this condition. *SHOC2* encodes a scaffold protein that positively modulates RAS-MAPK signal flow. The mutation was documented to promote *N*-myristoylation of the protein, and to drive aberrant targeting of SHOC2 to the plasma membrane and increased ERK activation in a cell context-specific fashion [Cordeddu et al., 2009]. The phenotype of these subjects is characterized by facial features that are reminiscent of NS, short stature, cognitive deficits, distinctive hyperactive

Disclosure summary: the authors have nothing to declare.

*Correspondence to:
M. Salerno, M.D., Ph.D., Department of Pediatrics, "Federico II" University of Naples, Napoli, Italy. E-mail: salerno@unina.it

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behavior, cardiopathy, and easily pluckable, sparse, thin, slow-growing hair in the anagen phase but lacking an inner and outer root sheaths. In most of these patients, short stature is mainly associated to proven GH deficiency (GHD) [Cordeddu et al., 2009].

We report on a patient with molecularly confirmed NS/LAH in whom severe short stature was found to be associated to GH insensitivity. Peculiar neurologic manifestations were also observed.

CLINICAL REPORT

The patient was born preterm (34 weeks of gestation) with a pregnancy complicated by polyhydramnios. Parents were not consanguineous. Birth weight was 2.450 kg (75th centile), length was 45 cm (25th–50th centile). She was referred to our attention at the age of 7 years for short stature. Physical examination revealed macrocephaly, high forehead, epicanthal folds, ptosis, hypertelorism, high and narrow palate, pterigium colli, pectus excavatum with widely spaced nipples, and deep palmar and plantar creases. She had severe growth delay: weight was 13 kg (−4.4 SDS) and height was 97 cm (−5.7 SDS), with impaired linear growth velocity (3 cm/year). Bone age was delayed by 3 years. Karyotype was normal (46, XX). Neurological evaluation revealed a mild psychomotor delay (IQ: 52) with impairment of verbal and language abilities. Cerebral MRI showed hypoplastic corpus callosum. Based on these features, NS disease genes were screened (*PTPN11*, *KRAS*, *SOS1*, *MEK1*, *BRAF*), but mutation analysis failed to reveal any causative mutation.

Biochemical evaluation of GH-IGF1 axis, performed to investigate the cause underlying short stature, revealed low serum levels of IGF1 (27 ng/ml, nv 60–350) and normal GH peak after arginine stimulation (16 ng/ml), suggesting a condition of mild GH insensitivity (GHI).

The patient underwent a therapeutic trial with recombinant human (rh) GH at a mean dose of 45 μg/kg/die. However, no significant improvement in linear growth was observed. Nevertheless, an arrest of growth velocity was observed when GH treatment was stopped. Thus, GH was re-started in order to normalize growth velocity (Fig. 1).

Over time, several ectodermal abnormalities became evident, including ungueal dystrophy, dry, fine textured, sparse hair, which were easily pulled from the scalp, and dry, hairless, darkly pigmented skin (Fig. 2). Such a distinctive association prompted *SHOC2* mutation analysis which confirmed the presence of the disease-causing mutation (c.4A>G, p.Ser2Gly), thus confirming diagnosis of NS/LAH.

At 9 years, she suddenly presented with complex motor and vocal tics characterized by repetitive stereotyped movements predominantly involving facial and shoulder muscles and repetitive sounds, which persisted continuously for more than 1 year. Major causes of secondary tics disorders in childhood were excluded, including occurrence of neurodegenerative disease, central nervous system infections, pediatric autoimmune neuropsychiatric disorder associated with streptococcal infection (PANDAS) or drug-related effects [Dooley, 2006]. A few months later, she presented with a generalized convulsive event. Electroencephalography revealed the presence of focal abnormalities in both parietal and occipital areas,

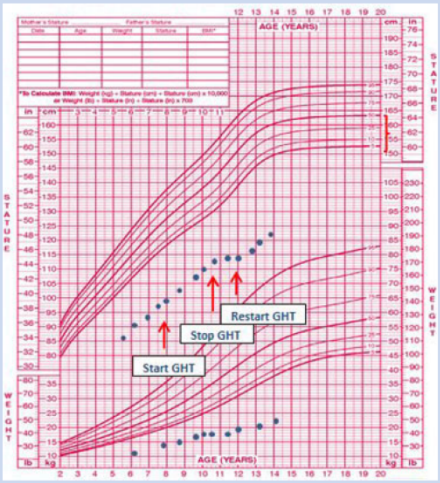

FIG. 1. Growth chart of the patient before and during GH therapy (GHT). GHT did not significantly improve growth velocity, however, stop of GHT was followed by an arrest of growth velocity and therefore GHT was re-started in order to normalize growth velocity.

whereas MRI confirmed the previous finding of corpus callosum hypoplasia and showed diffuse reduction in the white matter. She was then treated with carbamazepine with a good control on seizures but without any effect on tic disorders. She is now 13 years old. Despite 6 years of GH treatment, she shows a severe short stature (height −5 SDS) (Fig. 1), and IGF1 levels are still below the normal range (93.5 ng/ml; nv 180–780).

MATERIALS AND METHODS

IGF1 Generation Test

IGF1 generation test involved daily sc injections of 0.33 μg/kg/die rhGH for four consecutive days. Blood serum for IGF1 was taken before the first GH injection as well as on day 5. Serum IGF1 levels were measured using a two-site IRMA kit (Diagnostic System Laboratories, Inc., Webster, TX).

SHOC2, *GHR*, and *SOCS2* Mutational Analysis

Since the most common form of GHI is associated with genetic defects of the GH receptor (GHR; OMIM: 600946) mutation scanning of the gene was performed. Moreover, since the suppressor of cytokine signaling (SOCS) protein family, particularly SOCS2 (OMIM: 605117), act as negative regulators of GH signaling, *SOCS2* mutation screening was also carried out. Analyses were performed by direct sequencing as previously reported [Metcalf

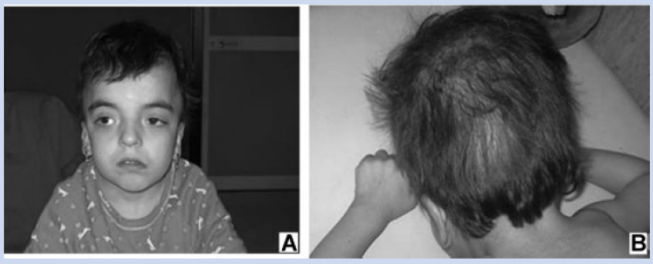

FIG. 2. The patient at 7 years of age. A: Frontal view: macrocephaly, high forehead, epicanthic folds, palpebral ptosis, hyperthelorism, high and narrow palate; (B): Back view: loose anagen hair.

et al., 2000]. Direct sequencing of the first exon of *SHOC2* was conducted as reported by Cordeddu et al. [2009].

GHR-Induced Phosphorylation of STAT5

The intracellular signaling elicited by GHR was studied through the analysis of GH-induced tyrosine phosphorylation of signal transducers and activators of transcription (STAT) 5, which was evaluated on peripheral blood mononuclear cells (PBMC). Briefly, cells were isolated by Ficoll-Hypaque (Biochrom) density gradient centrifugation using standard procedure, and cells were stimulated with GH (500 ng/ml, 5–15 min). PBMCs were lysed in 100 µl of lysis solution (20 mM Tris, pH 8, 137 mM NaCl, 1% Nonidet P-40, 10 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 5 µg/ml leupeptin, and 5 µg/ml aprotinin) on ice for 45 min. Lysates were centrifuged at 14,000*g* for 10 min at 4°C. Protein concentration of the cleared supernatants was determined by Bio-Rad protein assay. Lysates were stored at −80°C for WB analysis. Equal amounts of protein (12 µg) were resolved by electrophoresis on 10% denaturing polyacrylamide gel. Proteins were electro-transferred onto nitrocellulose transfer membranes, which were blocked in 10% bovine serum albumin in Tris-buffered saline-Tween-20 (TBST). Blocked membranes were reacted with rabbit polyclonal antibody anti-STAT5 (sc-835, Santa Cruz Biotechnology, Santa Cruz, CA) and anti pSTAT5 (sc-11761, Santa Cruz Biotechnology). After washings in TBST, the membrane was incubated with goat anti-rabbit polyclonal antibody peroxidase-linked (NA 934, Amersham, Little Chalfont, England) for 1 hr at room temperature. β-Actin was used as a loading control. ECL detection system was used for visualization.

RESULTS

IGF1 Generation Test

IGF1 generation test revealed only a mild increase of IGF1 levels (55 ng/ml) after stimulation with exogenous GH with respect to basal values (30 ng/ml). Moreover, the peak value obtained was still below the mean value for age (60–350 ng/ml).

SHOC2, *GHR*, and *SOCS2* Mutational Analysis

Direct sequencing of *SHOC2* exon 1 allowed the identification of the heterozygous condition for the A-to-G missense substitution at position 4, which predicts the p.Ser2Gly change in the encoded protein, confirming NS/LAH diagnosis. The molecular analysis of the entire coding sequence of the *SOCS2* and *GHR* genes failed to reveal causative mutations. The following known polymorphic changes were observed: rs1498708 of *SOCS2* (5′ untranslated region), rs6179 and rs6180, exon 6 and exon 10 of *GHR*, respectively.

GH-R-Induced Phosphorylation of STAT5

WB analysis of PBMC lysates showed that expression of STAT5 in the patient was comparable to that of healthy control (Fig. 3). Moreover, the phosphorylation level of the protein was comparable to that observed in control cells, suggesting normal GH-induced phosphorylation of STAT5 (Fig. 3).

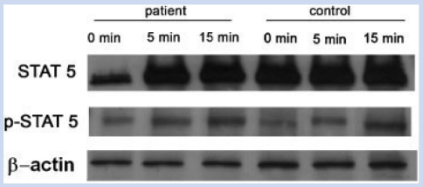

FIG. 3. Western blot analysis of proteins isolated from PBMCs of patient and healthy control. Cells were stimulated with 500 ng/ml recombinant GH for 5 and 15 min. Blots were probed with polyclonal antibodies against STAT5 protein (92 kDa). Equivalent loading was controlled by reprobing the membrane with β-actin. The protein in the patient was comparable with the control both baseline (0 min) and after treatment (5 and 15 min).

DISCUSSION

We report on a patient with molecularly confirmed diagnosis of NS/LAH exhibiting GH insensitivity and unusual neurological features.

Short stature is a common feature in NS/LAH, and is commonly associated with GHD. Although GHI has been previously reported in patients with RAS-MAPK dysregulation, only a few studies have investigated GH-IGF1 axis in these patients. GHI is a condition characterized by the peripheral resistance to GH action [Rosenfeld et al., 1994]. Transduction of the signal elicited by GHR is mediated by the JAK/STAT pathway. Tyrosine phosphorylation of JAK2 and STAT5 plays a crucial role in such a process, which ultimately results in gene transcription [Hwa et al., 2011]. The most common form of GHI is due to *GHR* mutations; other forms are caused by abnormalities in signaling cascade downstream GHR and in particular in STAT5b [Salerno et al., 2001; Hwa et al., 2011].

In our patient, *GHR* mutation analysis failed to reveal any sequence abnormality. Consistently, normal phosphorylation of STAT5 following GH stimulation in PBMCs suggests normal transducing properties of GHR. Based on recent findings supporting the existence of a dual negative action of SOCS2 on both GH and IGF1 independent signaling [Flores-Morales et al., 2006], we evaluated possible *SOCS2* involvement. However, mutation scanning of *SOCS2* did not reveal any sequence change with functional relevance.

Even if we failed to reveal alterations in *GHR*, *SOCS2* or GH-induced phosphorylation of STAT5, it is possible that the *SHOC2* mutation might directly alter GHR signaling through dysregulation of RAS-MAPK signaling. It has been well documented that NS patients with mutated *PTPN11* have features of GH resistance with lower levels of IGF1 and higher GH secretion compared to NS individuals without *PTPN11* mutations [Binder et al., 2005]. It is thought that enhanced phosphatase activity of SHP2, the protein encoded by PTPN11, negatively regulates GHR-JAK2-STAT5 signaling through an increased tyrosine phosphatase action [Stofega et al., 2000]. However mild features of GH insensitivity have been also detected in NS patients without *PTPN11* mutations [Ferreira et al., 2005], suggesting that also dysregulation in other molecules involved in RAS-MAPK might be responsible of GH insensitivity.

Our patient also showed atypical neurologic manifestations. In fact, in addition to a mild psychomotor delay and epilepsy, the patient developed complex chronic vocal and motor tics. These hyperkinetic disorders have not been previously reported in patients with *SHOC2* mutations nor in any other condition due to RAS-MAPK dysregulation. The precise etiology of chronic tics in childhood often remains elusive. However, abnormalities in the dopamine pathways have been supposed to be one of the possible mechanisms underlying neurotransmitter alterations [Du et al., 2010]. Furthermore, recent evidence suggests that RAS-MAPK pathways are involved in signal transduction activated by dopamine receptors [Zhen et al., 2010]. Thus, one could speculate that in our patient dysregulation of RAS-MAPK due to dysregulated SHOC2 function might be related to tics disorders through an alteration of dopamine-signaling. In keeping with this, studies on Gilles de la Tourette syndrome (GTS), one of the most frequent causes of chronic tics in infancy, also support a potential role of a cytokine-mediated immune activation in the pathogenesis of tics and other hyperkinetic disorders [Martino et al., 2009] suggesting that also an alteration of cytokine-signaling mediated by RAS-MAPK might be implicated in the pathogenesis of movement disorders in our patient.

In conclusion we report on a patient with NS/LAH due to the invariant c.4A>G mutation in *SHOC2*, with mild GH insensitivity and chronic tics. The extensive evaluation of GH-IGF1 axis failed to show abnormalities in GH receptor or STAT5 mediated post-receptorial signaling. Whether RAS-MAPK dysregulation induced by *SHOC2* mutation may interfere with GH-IGFI axis and neurotransmitter signaling still remains to be clarified.

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§ 3.1.1 Potential oncogenic role of the γc

§ 3.1.1.1 The γc provides spontaneous or induced cell proliferation

The intrinsic property of γc in cell cycle progression has been long debated. In fact, although gene therapy trials have been proved as a beneficial alternative approach to cure X-SCID patients carrying mutations of γc, a malignant lymphoproliferation occurred in 5 out of 20 patients enrolled into the two different trials [150,151], not observed in gene therapy trials for SCID due to ADA deficiency [152]. To explain these adverse events, studies were conducted to define whether the retroviral insertional mutagenesis could have played a role. In 4 cases an aberrant transcription and expression of LMO2 was clearly documented [153]. Even though the other patients may have the vector integration near LMO2 or other oncogenes [153], it is also conceivable that the transgene could have a role *per se* in cell cycle progression. Of note, development of leukemia, similar to other cancers, requires multiple genetic changes caused by a diverse group of genes that inhibit apoptosis and/or provide growth advantage to the leukemic cells [154]. In keeping with this hypothesis, overexpression of γc transduced through a lentiviral vector into stem cells in murine model of X-SCID led to T-cell lymphomas and thymic hyperplasia in a third of the cases. Intriguingly, no common integration site was found between the mice, which developed T-cell lymphomas [155]. In these mice, differently from humans treated with gene therapy for X-SCID, the expression levels of the protein was elevated, thus implying that the amount of the protein may be crucial for the γc control of cell cycle [156]. These results suggest that insertional mutagenesis may not be the only cause of laeukemogenesis and that the expression levels of γc could influence the cell cycle progression directly or its effect being mediated by cytokines triggers.

It has been documented that γc receptors activity enhances leukemogenesis [157]. To define an intrinsic mitogenic property of γc dependent on the amount of the protein, we used in vitro cellular models containing different amounts of γc.

In particular, EBV-transformed B-cells (BCLs) from normal subjects, cells transduced with lipid vector containing nontargeting short interfering RNA (siRNA), BCLs transduced with siRNA to knockdown γc expression and BCLs from X-SCID patients were used. Our results indicate that silencing of γc induces a substantial decrease of protein amount in BCLs, which allowed us to demonstrate a direct involvement of γc in self-sufficient growth of BCLs in a concentration dependent manner. We, also, found that the amount of constitutively activated JAK3 parallels the extent of γc expression. This finding is intriguing, in that constitutively active or hyperactive JAK proteins have crucial roles in hematopoietic malignancies, by promoting oncogenic transformation [157]. In particular, JAK overexpression can be considered as one of the main biologic events leading to the constitutive activation of the JAK/STAT pathway that contributes to oncogenesis [158]. In lymphoid cells, the involvement of the JAK/STAT pathway in several cellular processes, such as proliferation and protection from apoptosis, has also been well documented [159,160].

We found that γc silencing also inhibits GH-induced cell proliferation. In this context, it is known that the activation of JAKs and STATs represents a prominent biochemical event during GH-dependent proliferation of lymphoid cell lines [161-165] and STAT5 is considered a transforming agent in lymphoma and other cell types [166], therefore we found that the reduction of γc amount also inhibits STAT5 activation and its subsequent nuclear translocation, which follows GHR perturbation. Of note, it should be mentioned that experimental studies document a role for GH in the initiation and/or promotion of tumorigenesis, raising the possibility that patients treated with GH might be at increased risk of cancer [167]. Moreover, a putative role of GH as a cofactor in tumor growth is plausible, since several carcinomas express GHR [168]. In animal models, GH increase the incidence of leukemia and solid tumors, and in humans, at supraphysiological doses, it can promote lymphoproliferative events [169].

In conclusion, our data demonstrate a direct relationship between the amount of γc expression and its role in cell cycle progression. These data add new evidence

for a possible intrinsic mitogenic role of γc related to its cellular amount. This biologic effect could be direct, thus related to the molecule *per se*, or indirect and mediated by the participation to cytokine-receptors signaling. Therefore, since results of gene therapy trials for X-SCID have been very promising, to achieve safer results, the modulation of the transgene expression could help reduce the risk of undesirable events.

These data have been published as Article on *The Journal of Immunology*, for the manuscript see below.

The Cellular Amount of the Common γ-Chain Influences Spontaneous or Induced Cell Proliferation[1]

Stefania Amorosi,* Ilaria Russo,* Giada Amodio,* Corrado Garbi,† Laura Vitiello,†‡ Loredana Palamaro,* Marsilio Adriani,* Ilaria Vigliano,* and Claudio Pignata[2]*

Mutations of the *IL2RG* encoding the common γ-chain (γ_c) lead to the X-linked SCID disease. Gene correction through ex vivo retroviral transduction restored the immunological impairment in the most of treated patients, although lymphoproliferative events occurred in five of them. Even though in two cases it was clearly documented an insertional mutagenesis in *LMO2*, it is conceivable that γ_c could have a role per se in malignant lymphoproliferation. The γ_c is a shared cytokine receptor subunit, involved also in growth hormone (GH) receptor signaling. Through short interfering RNA or using X-linked SCID B lymphoblastoid cell lines lacking γ_c, we demonstrate that self-sufficient growth was strongly dependent on γ_c expression. Furthermore, a correlation between γ_c amount and the extent of constitutive activation of JAK3 was found. The reduction of γ_c protein expression also reduced GH-induced proliferation and STAT5 nuclear translocation in B lymphoblastoid cell lines. Hence, our data demonstrate that γ_c plays a remarkable role in either spontaneous or GH-induced cell cycle progression depending on the amount of protein expression, suggesting a potential role as enhancing cofactor in lymphoproliferation. ***The Journal of Immunology,*** **2009, 182: 3304–3309.**

Mutations of the *IL2RG* gene encoding the cytokine receptor common γ-chain (γ_c)[3] lead to the X-linked SCID (X-SCID) disease (1, 2). The severity of this disease makes it a medical emergency, which without any treatment leads to death in the first months of life. Bone marrow transplantation represents in this context the conventional therapeutic strategy for this form of immunodeficiency. This therapeutic approach confers to children affected by SCID at least a 70% chance of cure in the presence of a fully HLA-matched donor. Unfortunately, a fully compatible donor is not always available, thus limiting the successful use of this therapy. Moreover, the use of a not fully HLA-matched donor increases the immunologic complications such as graft-vs-host disease associated with a potential long-term decline in immune cell functions. These difficulties encouraged gene therapy trials (3). This strategy using ex vivo retroviral vectors has been proven as a corrective therapeutic approach for X-SCID in humans (4–9). Immunological reconstitution has been documented in 17 of 20 patients enrolled in two distinct clinical studies (3, 7). Unfortunately, five of these patients developed a lymphoproliferative disorder (10–12), not observed in gene therapy trials for SCID due to adenosine deaminase deficiency (13). This event was attributed to up-regulated expression of the *LMO2* oncogene, as a consequence of insertional mutagenesis (14). However, this event was clearly documented only in two cases. Even though the other patients may have the vector integration near *LMO2* or other oncogenes (14), it is also conceivable that the transgene could have a role per se in cell cycle progression. In keeping with this hypothesis, overexpression of γ_c transduced through a lentiviral vector into stem cells in a murine model of X-SCID led to T cell lymphomas and thymic hyperplasia in a third of the cases. Intriguingly, no common integration site was found between the mice, which developed T cell lymphomas (15). In these mice, differently from humans treated with gene therapy for X-SCID, the expression levels of the protein was elevated thus implying that the amount of the protein may be crucial for the γ_c control of cell cycle (16). These results suggest that insertional mutagenesis may not be the only cause of leukemogenesis and that the expression level of *IL2RG* could influence the cell cycle progression directly or its effect being mediated by cytokines triggers.

The γ_c is a transducing element shared among several IL receptors, whose activity was documented to enhance leukemogenesis (17), and is part of the intermediate- and high-affinity receptor of IL-2, that is essential for ligand internalization (18). In turn, this subunit activates several key signaling molecules such as JAK3, in which constitutive activation is frequently associated to autonomous cell growth and malignant transformation of lymphoid cells (19, 20). Recently, we demonstrated that γ_c subunit is also involved in growth hormone (GH) receptor (GHR) signaling in B lymphoblastoid cell lines (BCLs) (21). GH in BCLs obtained from X-SCID patients was unable to induce cell proliferation and STAT5 activation (22). *IL2RG* gene transduction of X-SCID BCLs promptly restored these functional and biochemical events, eventually resulting in STAT5 nuclear translocation (21).

In this study, we show through γ_c silencing experiments that the molecule is actively involved in a concentration dependent manner in self-sufficient growth and GH-induced cell cycle progression of BCLs, its activation being mediated by STAT5 phosphorylation and nuclear translocation.

*Department of Pediatrics, †Department of Cellular and Molecular Biology, and Pathology "Federico II" University, Naples, Italy; and ‡Department of Medical Science and Rehabilitation, Instituto di Ricovero e Cura a Carattere Scientifico San Raffaele Pisana, Rome, Italy

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[2] Address correspondence and reprint requests to Dr. Claudio Pignata, Professor of Pediatrics, Department of Pediatrics, Unit of Immunology, "Federico II" University, via S. Pansini 5-80131, Naples, Italy. E-mail address: pignata@unina.it

[3] Abbreviations used in this paper: γ_c, common γ-chain; GH, growth hormone; GHR, GH receptor; BCL, B lymphoblastoid cell line; siRNA, small interfering RNA; X-SCID, X-linked SCID.

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Materials and Methods

Reagents

Recombinant human GH was obtained from Serono. The ECL kit was purchased from Amersham Biosciences. The Abs anti-γ_c, anti-JAK3, anti-β-actin, anti-histone 3 (H3), anti-phosphotyrosine, anti-STAT5 were purchased from Santa Cruz Biotechnology. The neutralizing anti-IL-2 and anti-IL-4R mAbs were purchased from R&D Systems. Acrylamide and bisacrylamide were obtained from Invitrogen. Prestained molecular mass standards were obtained from Bio-Rad. The small interfering RNA (siRNA) duplexes specific for γ_c and the control nontargeting siRNA were obtained from Invitrogen. The control nontargeting pool contains nontargeting siRNAs with guanine cytosine content comparable to that of the functional siRNA but lacking specificity for known gene targets. Except where noted, other reagents were from Sigma-Aldrich.

Cells and cell cultures

Mononuclear cells (PBMC) were obtained from four X-SCID patients and six normal donors of heparinized peripheral blood by Ficoll-Hypaque (Biochrom) density gradient centrifugation (21). BCLs were generated by EBV immortalization of patients and control PBMC using standard procedures. Cells were maintained in RPMI 1640 (Biochrom) supplemented with 10% FBS (Invitrogen), 2 mM/L L-glutamine (Invitrogen), and 50 μg/ml gentamicin (Invitrogen), and cultured at 37°C, 5% CO_2. In self-sufficient growth experiments, BCLs were cultured in DMEM/F12 without FBS and supplemented with 2 mM/L L-glutamine.

In neutralization experiments, BCLs were cultured in 96-well plates, preincubated with the neutralizing mAbs 202 or 230 at the indicated concentrations.

siRNA transfection

Preparation of the cells before Lipofectamine 2000 transfection was performed according to the manufacturer's recommendations. Briefly, for each transfection 1×10^6 BCLs in 1 ml were treated with 20 μl of 50 μM siRNAs specific for the γ_c or equal amount of the control nontargeting siRNA. The siRNAs were solubilized and formed complexes separately with the lipid-based transfectant, Lipofectamine 2000. The siRNA-lipofectamine complexes were transfected into the cultured cells in a 24-well plate and incubated for the time indicated in the text. Throughout the experiments, cell vitality was monitored continuously by trypan blue exclusion assay. Furthermore, 96 h after the transfection, the cells were washed, placed in fresh culture medium and used for further analysis, as described.

CFSE labeling

Cell proliferation was measured by the cell surface stain CFSE. BCLs (1×10^6) were labeled with 1.7 μM CFSE in PBS just before culturing for the indicated times using a serum-free medium. After 2 min at room temperature, BCLs were washed in FBS and PBS and cell division accompanied by CFSE dilution was analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences).

[^{3}H]thymidine incorporation assay

Cell number was assessed by counting cells after trypan blue dye exclusion staining. BCLs were cultured for different time ranging between 6 h and 4 days at a density of 1×10^5 viable cells/200 μl well in triplicate wells (96-well microtiter plates, Falcon; BD Biosciences). Cultures were pulsed with 0.5 μCi [^{3}H]thymidine for 8 h (or 6 h in the short-term cultures) before harvesting and the incorporated radioactivity measured by scintillation counting. Where indicated (see Fig. 5), recombinant GH was added to the culture at 50 ng/ml. The results are expressed as mean cpm for triplicate cultures.

Immunoprecipitations and Western blotting

Following transfection and appropriate recombinant GH stimulation, BCLs were lysed in 100 μl of lysis solution (20 mM Tris (pH 8), 137 mM NaCl, 1% Nonidet P-40, 10 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 5 μg/ml leupeptin, and 5 μg/ml aprotinin) on ice for 45 min. Protein concentration was determined by Bio-Rad protein assay. The cell lysates were stored at −80°C for Western blot analysis. Nuclear extracts were prepared by the method previously described (21). Proteins were electrophoretically separated on 10% Tris glycine SDS-PAGE gels. Proteins were transferred onto nitrocellulose transfer membranes (Schleicher & Schuell). Membranes were incubated with the specific primary Abs. Immune complexes were detected using the appropriate anti-rabbit or anti-mouse peroxidase-linked Abs. ECL detection system was used for visualization.

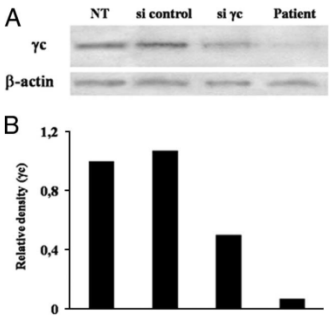

FIGURE 1. The γ_c silencing by siRNA induced a reduction in protein amount. *A*, After 96 h of culture, control BCLs transfected with nontargeting siRNA (si control), γ_c siRNA (si γ_c), or nontransfected (NT), and X-SCID BCLs (patient) were lysed and γ_c total amount was measured by Western blotting. Membranes were incubated as indicated with Abs anti-γ_c and anti-β-actin, used as loading control. *B*, Densitometric analysis of the above Western blot. ImageJ program was used to generate the data.

Equal loading was confirmed after stripping and reprobing with anti-β-actin or anti-histone 3 Abs.

For immunoprecipitation, lysates were normalized for either protein content or cell number and precleared with protein G-agarose beads (Amersham Biosciences). The supernatant was incubated with 2 μg/ml anti-JAK3 or polyclonal serum, followed by protein G-agarose beads. The immunoprecipitates were separated on density gradient gels, followed by Western blotting. Proteins were detected using Ab for phosphotyrosine.

Densitometric analysis was performed on a Windows personal computer, using the public domain Java image processing program ImageJ (developed at the National Institutes of Health and at (http://rsb.info.nih.gov/ij/index.html). Each signal has been evaluated in comparison with the control lane 1 and equalized for the loading control, applying the following formula: (sample lane/control lane)/loading control ratio.

Confocal microscopy

After appropriate stimulation, as indicated, cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.0) for 30 min at room temperature and centrifuged in a Shandon Cytospin III (Histotronix) onto a glass slide and permeabilized by incubation in a 0.2% Triton X-100 solution for 20 min (21). BCLs were incubated for 1 h at room temperature with rabbit anti-STAT5 Ab in PBS containing 1% BSA. After four washings for 5 min in PBS, the cells were incubated for 1 h at room temperature with FITC-conjugated donkey anti-rabbit IgG (Pierce) in PBS. After washing in PBS, the glass slides were mounted under a coverslip in a 5% glycerol PBS solution. The slides were analyzed by laser scanning confocal microscopy using a Zeiss LSM 510 (version 2.8 SP1 Confocal System). At least 100 cells per condition were analyzed in each experiment to determine the rate of STAT5 nuclear translocation.

Results

Common γ_c silencing inhibits self-sufficient growth and down-regulates constitutively activated JAK3 in B cell lines

To define an intrinsic mitogenic property of γ_c dependent on the amount of the protein, we used in vitro cellular models containing different amounts of γ_c. In particular, BCLs from normal subjects, cells transduced with lipid vector containing nontargeting siRNA, BCLs transduced with siRNA to knockdown γ_c expression and BCLs from X-SCID patients were used. The transfection efficiency was tested using fluorescent oligonucleotides under fluorescent microscope. Levels of γ_c were evaluated by Western blotting of whole cell lysates. The γ_c expression was reduced to 50% of the control in γ_c-silenced BCLs and completely undetectable in X-SCID BCLs (Fig. 1*A*). Densitometric analysis is shown in the histogram in Fig. 1*B*.

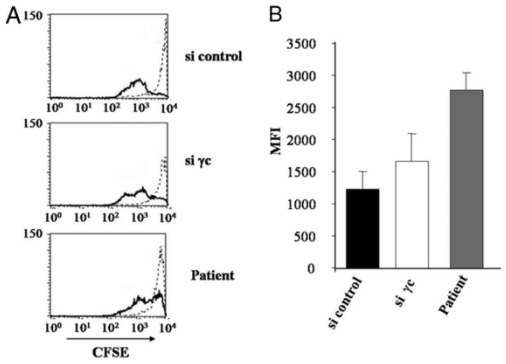

FIGURE 2. The γ_c was involved in self-sufficient growth of BCLs. *A*, After 12 h of starvation, BCLs transfected with control nontargeting siRNA (si control) or γ_c siRNA (si γc) and X-SCID BCLs (patient) were stained with 1.7 μM CFSE and cultured in the absence of serum. After 6 h, cells were harvested and cell proliferation was assessed using cytofluorimetry for CFSE intensity. CFSE dilution profiles are shown. Histograms show on gated cells the number of events (*y*-axis) and the fluorescence intensity (*x*-axis) 6 h following the start of the culture. Dashed lines represent the start of the culture. *B*, Mean fluorescence intensity (MFI) of gated CFSE-positive cells maintained in the same conditions as described in *A*.

Because self sufficiency in growth has been suggested as one of the six acquired capabilities of cancer phenotype (23), we examined the abilities of these previously mentioned BCLs to grow in serum-deficient conditions, upon trypan blue exclusion assay. We labeled BCLs, after 12 h of starvation, with CFSE, a dye that allows proliferative history to be visualized, and assessed the

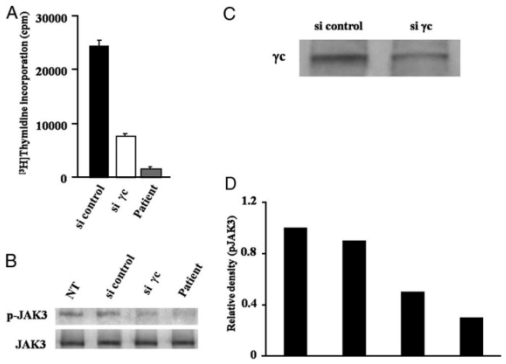

FIGURE 3. The γ_c protein depletion had effect on spontaneous cell proliferation and on activated JAK3 levels. *A*, BCLs transfected with control nontargeting siRNA (si control) or γ_c siRNA (si γc) and X-SCID BCLs (patient) were tested for their ability to proliferate in serum-free medium. Cultures were maintained in serum-free medium for 4 days and pulsed with [^{3}H]thymidine for the final 8 h. Radioactive incorporation was counted. Error bar indicates 1 SD. *B*, Unstimulated BCLs, after 12 h of starvation, were immunoprecipitated with anti-JAK3 Ab and tested in Western blot with anti-phosphotyrosine mAb. Equivalent loading was controlled by reprobing the membrane with JAK3 Ab. Nontransfected (NT) BCLs were also tested. *C*, Control BCLs, transfected with nontargeting siRNA (si control) and γ_c siRNA (si γc), were lysed and γ_c total amount was measured by Western blotting. *D*, Densitometric analysis of Western blot shown in *B*.

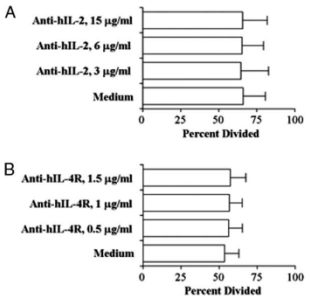

FIGURE 4. The γ_c-activating cytokines did not affect self-sufficient growth of BCLs. *A*, Control BCLs were treated with the indicated concentration of Anti-human IL-2, were stained with 1.7 μM CFSE and cultured in the absence of serum. After 6 h, cells were harvested and cell proliferation was assessed using cytofluorimetry for CFSE intensity. *B*, Control BCLs were treated with the indicated concentration of anti-human IL-4R and analyzed as described in *A*. The percentage of cells that divided is shown. Error bar indicates 1 SD.

CFSE dilution profile at different time points ranging between 6 h and 7 days to establish the rate of spontaneous cell proliferation (data not shown). Informative data on differences between previously described BCLs were appreciable as soon as 6 h from the start of the culture, presumably because of the high proliferation rate of BCLs as compared with normal mononuclear cells. At this time, only 14% of control cells retained the dye, indicating a high proliferation rate, compared with 26% of γ_c-silenced cells and to 50% of X-SCID BCLs (Fig. 2*A*). In addition, the final mean fluorescence intensity, reflecting CFSE-derived fluorescence per cell, were 1378 units in control, 1825 in γ_c-silenced, and 2866 in X-SCID, thus confirming that only in control cells a substantial dye dilution occurred (Fig. 2*B*).

We then cultured viable cells for 4 days in a serum free medium and 0.5 μCi of [^{3}H]thymidine were added 8 h before harvesting. γ_c-silencing reduced cell proliferation of unstimulated BCLs by 69% as compared with control cells. In X-SCID BCLs the extent of the reduction was higher corresponding to 97% of control BCLs (Fig. 3*A*). These data were, therefore, in keeping with the results of CFSE experiments. Moreover, to prove that the effect observed in the CFSE experiments in the 6 h cultures were really indicative of cell proliferation, several time-course experiments with both techniques were performed at the beginning of the study. These data indicate that the proliferation rate of these cells is comparable using the two methods in the first 12 h (see supplemental materials S1 and S2),[4] indicating that the CFSE dilution reflects a real cell division. Furthermore, the addition of mitomycin prevents staining dilution, providing further evidence that CFSE signals reflect a real cell division. In particular, after 6 h of culture 12, 31, and 59% of the control, silenced or SCID patient cells, respectively, retained the dye as compared with the 100% of stained cells at the beginning of the culture (see supplemental material S3).[4]

Because JAK3 is essential for autonomous proliferation being physically linked to γ_c, we further investigated the role of γ_c in self-sufficient growth, evaluating JAK3 activation. Of note, JAK3 proteins are constitutively phosphorylated in EBV-immortalized B cells and other malignant cells (24). Thus, we evaluated the effect of different amount of γ_c on the levels of constitutively phosphorylated JAK3 protein (phospho-JAK3). After 12 h of serum-free

[4] The online version of this article contains supplemental material.

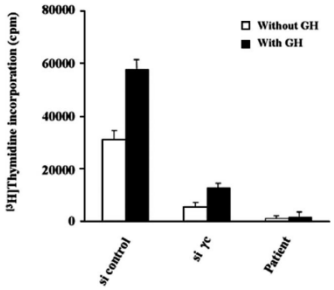

FIGURE 5. Silencing of γ_c inhibited GH-induced proliferation. Cell proliferation of BCLs transfected with control nontargeting siRNA (si control) or γ_c siRNA (si γc) or nontransfected (NT) stimulated with recombinant GH (50 ng/ml) was evaluated through [^{3}H]thymidine incorporation assay. Error bar indicates 1 SD.

culture, whole cell lysates were immunoprecipitated with anti-JAK3 Ab and the obtained membranes were immunoblotted with anti-phosphotyrosine mAb. A higher constitutive activation of JAK3 was found in control BCLs, whereas a decrease in phospho-JAK3 levels was observed in γ_c-silenced and in X-SCID BCLs, despite a comparable amount of the whole protein (Fig. 3*B*). The amount of pJAK3 paralleled the amount of γ_c, shown in Fig. 3*C*. The densitometric analysis of phospho-JAK3 equalized for total JAK3 is shown in a histogram in Fig. 3*D*.

Evidence is available that γ_c-dependent cytokines, as IL-2 and IL-4, may be secreted in EBV-infected B cells (25–27). Thus, to define whether the mitogenic effect of γ_c was independent or dependent from receptor engagement of these endogenous γ_c-activating cytokines, we used neutralizing mAbs anti-IL-2 or anti-IL-4R in the CFSE-based proliferative assay. As shown in the Fig. 4, the neutralizing mAbs did not reduce at any concentration spontaneous cell proliferation.

The γ_c silencing inhibits GH-induced cell proliferation and subsequent STAT5 activation

It has been reported that GH enhances BCLs proliferation in vitro (28) and that γ_c is functionally linked to GHR (21). Moreover, it has been described the association between lymphoproliferative events and supraphysiological doses of GH, both in mice and humans (29).

Because growth factors may participate in autocrine or paracrine loops that affect tumor cells growth or survival and autocrine production of GH is able to induce cellular transformation (30), we evaluated the response to GH stimulation of X-SCID BCLs, control cells and γ_c-silenced BCLs to assess whether γ_c amount could influence GH response. Recombinant GH at a concentration of 50 ng/ml enhanced proliferation of control BCLs. In γ_c-silenced or X-SCID BCLs, recombinant GH induced proliferation at a much lower extent, corresponding to 28% and 5% of the control, respectively (Fig. 5).

Because the activation of JAKs and STATs represents a prominent biochemical event during GH-dependent proliferation of lymphoid cell lines (31–35) and STAT5 is considered a transforming agent in lymphoma and other cell types (36), we then evaluated whether γ_c-silencing had effect on GH-induced STAT5 subcellular localization. Nuclear and cytoplasmic extracts from BCLs, unstimulated or treated with 500 ng/ml recombinant GH, were evaluated by immunoblot for the overall amount of STAT5. Recombinant GH induced a rapid decrease of the cytoplasmic amount of STAT5 in control BCLs and in BCLs treated with control nontargeting siRNA, differently from γ_c-silenced BCLs, in which no effect on the protein amount was observed (Fig. 6*A*). This finding was inversely correlated with the amount of the nuclear form of the molecule. In fact, in control BCLs and in BCLs treated with control siRNA, an increase of nuclear STAT5 amount was observed after recombinant GH stimulation, differently from what observed in γ_c-silenced BCLs, in which no change was observed (Fig. 6*C*). These data, representative of different experiments, reflect a real subcellular redistribution of the molecule in that no difference in the cytoplasmic β-actin and nuclear histone H3 expression was observed. The densitometric analysis normalized for the housekeeping molecules is shown in Fig. 6, *B* and *D*.

Furthermore, we looked at STAT5 subcellular localization using confocal microscopy. In control unstimulated BCLs, only 10% of cells showed a nuclear localization of STAT5, being the protein mainly concentrated in the cytoplasm. GHR perturbation through recombinant GH stimulation at a concentration of 500 ng/ml

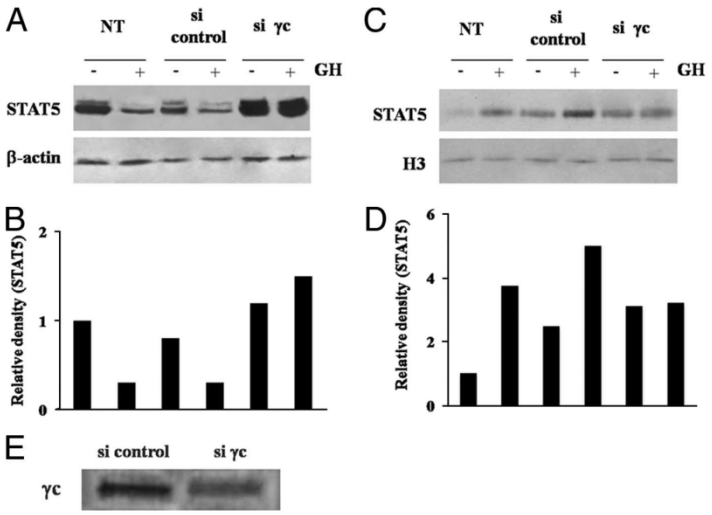

FIGURE 6. Silencing of γ_c influenced STAT5 nuclear translocation in B cell lines. Cells from BCLs transfected with control nontargeting siRNA (si control) or γ_c siRNA (si γc) and X-SCID BCLs (patient) were analyzed for subcellular localization of STAT5. Cells were stimulated with 500 ng/ml recombinant GH for 30 min. *A*, Cytoplasmic amount of STAT5. Equivalent loading was controlled by reprobing the membrane with β-actin. *C*, Nuclear fraction of STAT5. Equivalent loading was controlled by reprobing the membrane with histone H3. *B* and *D*, Densitometric analysis of the Western blots from BCLs in *A* and *C*. *E*, Control BCLs, transfected with nontargeting siRNA (si control) and γ_c siRNA (si γc), were lysed and γ_c total amount was measured by Western blotting.

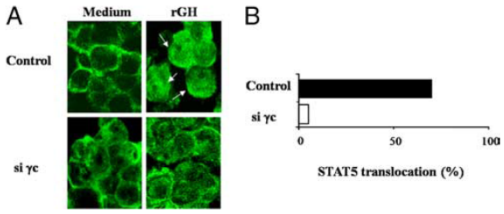

FIGURE 7. The γ_c silencing impairs recombinant GH-induced STAT5 subcellular redistribution. *A*, Evaluation of STAT5 subcellular localization through confocal microscopy. Control or γ_c-silenced BCLs (si γc) were cultured in the absence or presence of 500 ng/ml recombinant GH for 30 min. Arrows indicate exemplificative cells with nuclear STAT5 staining. Nucleoli are not stained. *B*, The percentage of STAT5 nuclear translocation is shown. These data represent an analysis of independent observations.

induced STAT5 nuclear localization in the 70% of cells. Differently, recombinant GH stimulation of γ_c-silenced BCLs had negligible effects on nuclear STAT5 migration, resulting in a 5% increase of positively stained cells as compared with unstimulated cells (Fig. 7).

Discussion

Our results indicate that silencing of γ_c induces a substantial decrease of protein amount in BCLs, that allowed us to demonstrate a direct involvement of γ_c in self-sufficient growth of BCLs in a concentration dependent manner. Moreover, we documented that the amount of γ_c also influences the response of BCLs to GH-induced proliferation and STAT5 subcellular redistribution that follows GHR perturbation. These data add new evidence for a possible intrinsic mitogenic role of γ_c related to its cellular amount. This biologic effect could be either direct, thus related to the molecule per se, or indirect and mediated by the participation to cytokine-receptors signaling.

The intrinsic property of γ_c in cell cycle progression has been long debated. In fact, although gene therapy trials have been proved as a beneficial alternative approach to cure X-SCID patients carrying mutations of γ_c, a malignant lymphoproliferation occurred in 5 of 20 patients enrolled into the trials, alarming the scientific community (3). To explain these adverse events, studies were conducted to define whether the retroviral insertional mutagenesis could have played a role. In two cases, an aberrant transcription and expression of *LMO2* was clearly documented (14). However, for the remaining patients there isn't any evident demonstration of *LMO2* alteration due to random insertions that could be causative in transformation. An in vivo expansion of cell clones has also been documented in other gene therapy trials. Two patients treated with gene therapy for X-linked chronic granulomatous disease developed myeloid proliferation. Of note, in these cases cell clones didn't exhibit any self-renewal capacity. This observation would imply that there is no evidence of continued abnormal growth of clones containing insertionally activated growth-promoting genes (37). Of note, development of leukemia, similar to other cancers, requires multiple genetic changes caused by a diverse group of genes that inhibit apoptosis or provide growth advantage to the leukemic cells (38). In this study, we demonstrate that γ_c exerts a role in cell cycle progression in a strictly concentration dependent manner. We, also, found that the amount of constitutively activated JAK3 parallels the extent of γ_c expression. This finding is intriguing, in that constitutively active or hyperactive JAK proteins have crucial roles in hematopoietic malignancies, by promoting oncogenic transformation and uncontrolled blood cell production (17). In particular, JAK overexpression can be considered as one of the main biologic events leading to the constitutive activation of the JAK-STAT pathway, that contributes to oncogenesis (20). In lymphoid cells, the involvement of the JAK/STAT pathway in several cellular processes, such as proliferation and protection from apoptosis, has also been well documented (39, 40). Moreover, the role of JAK3 in cell destiny is emphasized by the finding that JAK3 mutations cause a SCID phenotype, thus implying its role in lymphoid development (41). JAK3 has also the capacity to activate DNA synthesis and protooncogenes, such as c-*myc* and c-*fos* (42).

In this study, we also observed that the participation of γ_c in GHR signaling apparatus and, in particular, in GH-induced STAT5 activation and nuclear translocation was also dependent on the extent of its molecular expression. Thus, the concentration-dependent mitogenic effect of γ_c could be favored by the participation of γc in GHR signaling. Of note, it should be mentioned that experimental studies document a role for GH in the initiation or promotion of tumorigenesis, raising the possibility that patients treated with GH might be at increased risk of cancer (29). Moreover, a putative role of GH as a cofactor in tumor growth is plausible because several carcinomas express GHR (43). In animal models, GH increase the incidence of leukemia and solid tumors, and in humans, at supraphysiological doses, it can promote lymphoproliferative events (44).

Our data would imply that the expression levels of γ_c in hematopoietic cells are crucial for the maintenance of cell growth control. Whether our data may have direct implications in the understanding of the pathogenesis of the lymphoproliferative events occurring during gene therapy trials for X-SCID remains to be clarified. Even though, under ordinary conditions, γ_c is expressed at a normal extent in cells, transduced with retroviral vectors containing wild-type γ_c, our data indicate that altering the expression levels of the protein could be important in modifying cell cycle control mechanisms. Our findings are in keeping with a recent study, which demonstrates that in X-SCID murine model, T cell lymphomas and thymic hyperplasia occur in a third of the cases treated with lentiviral vectors containing wild-type γ_c (15). This event was independent from common integration sites and, thus, not attributable to insertional mutagenesis, but rather to an intrinsic oncogenic property of the transgene and, presumably, to the overexpression of the molecule (15). Furthermore, by searching in Mouse Retroviral Tagged Cancer Gene Database, integration in *Il2rg* has been found in two cases of retrovirally induced leukemias (45).

In conclusion, our data demonstrate a direct relationship between the amount of γ_c expression and its role in cell cycle progression. Therefore, because results of gene therapy trials for X-SCID have been very promising, to achieve safer results, the modulation of the transgene expression could help reduce the risk of undesirable events.

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Disclosures

The authors have no financial conflict of interest.

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§ 3.1.1.2 Role of γc on spontaneous cell cycle progression in human malignant cell-lines

Cell cycle progression is a highly organized and regulated process that controls cell proliferation [170]. Cytokines that signal through receptors sharing the γc lead to transition into the cell cycle and thus proliferation [171]. The entry of cells into the cell cycle is controlled by an ordered expression/activation of cyclins [172]. IL-2R through γc appears to activate a variety of downstream signaling pathways that converge on the regulation of Bcl-2 [173], including PI3K and MAPK activation [174] and transcription of the c-myc gene [173]. In turn, c-myc cooperates with STAT5 to induce the expression of cyclin D and to promote proliferation [175-177]. It is clear that alterations in Bcl-2 family members levels exert potent effects on cellular survival and, namely, Bcl-2 overexpression can be tumorigenic [178]. Moreover, γc is required for a wide range of signaling inputs that induce cell proliferation through cyclin D3 expression [179].

To determine whether γc deficiency had an effect on cell survival we examined BCLs from healthy donors and X-SCID patients. Our results first indicate that in the absence of γc expression, BCLs die at a higher extent than control cells. This phenomenon is not related to abnormalities of regulatory mechanisms of apoptosis, in that, in non-stimulated cells, the absence of cc does not induce an increase of the activated form of caspase-3 that represents the central executioner molecule in the development of programmed cell death process [180]. .

Programmed cell death is mainly mediated by activation of several caspases These molecules exist as pro-forms that are activated by cleavage by the upstream caspase in the cascade [180]. Caspase-independent cell death has been attributed to mitochondrial damage [181], which can be regulated by Bcl-2 family members [182,183]. The γc-dependent cytokines promote cell survival by up-regulating the antiapoptotic factor Bcl-2 [184] and Bcl-XL [185]. In keeping with this, in γc-deficient cells, the expression of Bcl-2 and Bcl-XL was greatly decreased as compared with the control. These findings indicate that γc is required for cell survival and is dispensable for Fas induced cell death. Evidence exists that

autophagy can plays an active role in cell death, by contributing to cell death in unfavourable settings such as nutrient or growth factors deprivation. In keeping with this, probably γc could have a role in the autophagy process.

To define whether the effect of γc on cell cycle progression is a peculiarity of lymphoblastoid cells or a more general phenomenon involved in cell growth of malignancies of hematopoietic cell lineages, in this study we evaluated whether γc expression could interfere in cell cycle progression in neoplastic cells. we observed that the amount of cc protein expression in several malignant cell lines directly correlates with spontaneous cell growth. We, also, found that the knockdown of γc molecule through siRNA is able to decrease the cell proliferation rate in these malignancies, thus confirming a direct involvement of the molecule as a key player in cell cycle progression.

Alterations in cell cycle machinery, mainly in the regulation of G1/S phase, are known to be associated with the development of solid tumors as well as hematological malignant diseases [186]. Of note, cyclins are the key regulators of cell cycle progression [172]. In particular, during the G0 to G1 phase transition, cyclins D1, D2 and D3 are the first molecules to be induced. Cyclin A2 gets activated during the transition from G1 to S phase and B type cyclins are detected during G2 exit and mitosis phase [187]. Namely, cyclins A2 and B1 have been implicated in the pathogenesis of cancer and are overexpressed in several tumors [188,189]. Evidence indicates that these cyclins are key components of the cell-cycle machinery [190] and, in particular, cyclin A is expressed at high levels in hematopoietic stem cells and is essential for their proliferation [191]. In our study, we observed that the expression of A2 and B1 cyclins strongly paralleled the proliferative capability of malignant cell lines. Interestingly, a positive correlation between the amount of γc and the expression of cyclins A2 and B1 was also found. Taken together these data indicate that the higher is the rate proliferation of a certain cell line the higher is the expression of both γc and cyclins A2 and B1, thus confirming their involvement in the process in a concentration dependent fashion. We also found an increased expression of all D-

type cyclins in those cell lines that proliferated mostly, K-562 and Molt-4, whereas they were not expressed in the other cell lines, but D1 found in Rj225.

D-type cyclins are strongly expressed in many malignancies. Overexpression of cyclin D1 protein was documented in many forms of cancer, including breast cancer [192], while overexpression of cyclin D2 was noted in a wide range of B cell malignancies, such as B cell chronic lymphocytic leukemia [193]. Like the other D cyclins, cyclin D3 is rearranged and the protein is overexpressed in several human lymphoid malignancies. It was documented that knockdown of cyclin D3 inhibits the proliferation of acute lymphoblastic leukemia cells [194]. However, while A and B type cyclins seem to be vital and necessary components of cell cycle progression [191], D-type cyclins may be dispensable for proliferation under certain circumstances, in that different cell types are sensitive to cyclin D knockdown at a different extent [195]. This would suggest that they regulate cell cycle in a cell-type specific manner and that there are alternative mechanisms allowing cell cycle progression in a cyclin D-independent fashion [195]. Anyway, a critical role for oncogenic transformation of D-type cyclins is a well established feature.

In conclusion, our data demonstrate that γc is strongly implicated in cell cycle progression of hematopoietic malignancies. This biological effect is dependent on the expression level of the molecule. Moreover, we documented that the IL-2Rγ mRNA levels was also highly expressed in primary leukemic cells, thus confirming a direct involvement of the γc in tumor growth.

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Role of the common γ chain in cell cycle progression of human malignant cell lines

Ilaria Vigliano[1], Loredana Palamaro[1], Gabriella Bianchino[2], Anna Fusco[1], Laura Vitiello[3], Vitina Grieco[2], Rosa Romano[1], Marco Salvatore[2,4] and **Claudio Pignata[1,*]**

[1]Department of Pediatrics, ''Federico II'' University, Naples 80131, Italy
[2]Molecular Oncology Unit, IRCCS, CROB, Rionero in Vulture, Pz, 85028, Italy
[3]Department of Cellular and Molecular Biology and Pathology and [4]Department of Biomorphological and Functional Sciences, Section of Radiology, ''Federico II'' University, Naples 80131, Italy

**Correspondence to*: C. Pignata; E-mail: pignata@unina.it

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Abstract

The γ-chain (γc) is a transducing element shared between several cytokine receptors whose alteration causes X-linked severe combined immunodeficiency. Recently, a direct involvement of γc in self-sufficient growth in a concentration-dependent manner was described, implying a direct relationship between the amount of the molecule and its role in cell cycle progression. In this study, we evaluate whether γc expression could interfere in cell cycle progression also in malignant hematopoietic cells. Here, we first report that in the absence of γc expression, lymphoblastoid B-cell lines (BCLs) die at a higher extent than control cells. This phenomenon is caspase-3 independent and is associated to a decreased expression of the antiapoptotic Bcl-2 family members. By contrast, increased expression of γc protein directly correlates with spontaneous cell growth in several malignant hematopoietic cell lines. We, also, find that the knockdown of γc protein through short interfering RNA is able to decrease the cell proliferation rate in these malignancies. Furthermore, an increased expression of all D-type cyclins is found in proliferating neoplastic cells. In addition, a direct correlation between the amount of γc and cyclins A2 and B1 expression is found. Hence, our data demonstrate that the amount of the γc is able to influence the transcription of genes involved in cell cycle progression, thus being directly involved in the regulatory control of cell proliferation of malignant hematopoietic cells.

Keywords: cell proliferation, cytokines, gamma chain, SCID

Introduction

The common γ-chain (γc) gene localized to chromosome Xq13 encodes a transmembrane protein which is a transducing element shared by the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 (1). Deficiency in the expression or function of the γc causes the X-linked severe combined immunodeficiency (X-SCID), characterized by the complete absence of both T and NK lymphocytes and normal B-cell number (2). It is known that the cytokines that act through the γc are generally growth factors (3). Upon cytokine interaction, IL-2R activates numerous downstream signaling molecules, including Janus kinases (JAK) and signal transducers and activators of transcription (STAT). In particular, STAT5, which is directly phosphorylated and activated by JAK3 (4), seems to play a major role in cell proliferation (5–7). γc signaling cytokines promote proliferation in T-cell acute lymphoblastic leukemia (ALL), thus implying a synergistic effect of these cytokines on tumor growth (8). It was also reported that *IL2RG* cooperates with *LMO2* in inducing hematopoietic tumors in X-SCID patients enrolled in gene therapy trials (9, 10).

Recently, we documented a direct relationship between the amount of γc expression and its role in cell cycle progression. In particular, using lymphoblastoid B-cell lines (BCLs), we described a direct involvement of γc in self-sufficient growth in a concentration-dependent manner. Furthermore, the γc amount correlated with the amount of constitutively activated JAK3, while a reduction of γc protein expression resulted in reduced STAT5 nuclear translocation in BCLs (11). This finding led to hypothesize that γc, through the modulation of genes related to growth signaling and cell cycle control, under certain circumstances plays a role as potential oncogene in tumor growth. However, whether γc is implicated in anti-apoptotic mechanism or directly involved in promoting cell signaling of cell cycle progression is not clear.

Cell cycle progression is a highly organized and regulated process that controls cell proliferation (12). Cytokines that signal through receptors sharing the γc lead to transition into the cell cycle and thus proliferation (3). The entry of cells into the cell cycle is controlled by an ordered expression/activation of

cyclins (13). IL-2R through γc appears to activate a variety of downstream signaling pathways that converge on the regulation of Bcl-2 (6), including PI3K and MAPK activation (14) and transcription of the *c-myc* gene (6). In turn, *c-myc* cooperates with STAT5 to induce the expression of cyclin D and to promote proliferation (15–17). It is clear that alterations in Bcl-2 levels exert potent effects on cellular survival and, namely, Bcl-2 overexpression can be tumorigenic (18). Moreover, γc is required for a wide range of signaling inputs that induce cell proliferation through cyclin D3 expression (19).

To define whether the effect of γc on cell cycle progression is a peculiarity of lymphoblastoid cells or a more general phenomenon involved in cell growth of malignancies of hematopoietic cell lineages, in this study, we evaluated whether γc expression could interfere in cell cycle progression in neoplastic cells. We also investigated whether the effect of γc is mediated by A2, B1, D1, D2 and D3 cyclins and is required for a proper activation of these cyclins in cell cycle progression.

Methods

Cell cultures

PBMCs were obtained from X-SCID patients and healthy donors by Ficoll-Hypaque (Biochrom, Berlin, Germany) density gradient centrifugation (20). Lymphoblastoid B-cell lines (BCLs) were generated by EBV immortalization of patients and healthy donors PBMC using standard procedures. The human T-ALL cell line (Molt-4), the chronic myelogenous leukemia cell line (K-562), Burkitt lymphoma cell line and its isogenic derivatives (Raji and Rj225) were grown in RPMI-1640 (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, California), 2 mmol L^{-1} L-glutamine (Gibco), and 50 μg ml^{-1} gentamycine (Gibco) and cultured at 37°C, 5% CO2. Serum starvation was used to synchronize tumor cells in the G0/G1-phase of the cell cycle. The cells were incubated in medium without FBS for 24 h. In self-sufficient growth experiments, cells were cultured in DMEM/F12 (Lonza) without FBS and supplemented with 2 mmol L^{-1} L-glutamine.

Primary leukemic cell lines, consisting of ALL cells, were obtained from aspirated bone marrow of three patients. Normal bone marrow cells were obtained from healthy donors and used as control cells.

Cell proliferation assays and cell survival

Cells were plated in triplicate at 1×10^5 viable cells/well in 96-well plates (BD Biosciences, San Jose, CA), in 200 μl of complete medium for 4 days. Cultures were pulsed with 0.5 μCi ^{3}H-thymidine for 8 h before harvesting and the incorporated radioactivity measured by scintillation counting. Cell proliferation was also analyzed by the CFSE dilution assay. Cells (1×10^6) were resuspended in 1 ml PBS-10% FBS and labeled with 1.7 μM CFSE (Molecular Probes, Leiden, Netherlands). After 2 min in the dark at room temperature, cells were washed in FBS and PBS. After 6 h, cells were analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences).

Cell viability was determined using trypan blue staining. Cell survival was evaluated following stimulation with anti-Fas mAb (400 ng ml^{-1}; Upstate, Lake Plasid, NY) for 6 h.

Western blot analysis

Stimulated or unstimulated cells were washed with ice-cold PBS (Cambrex, Charles City, IA) and lysed in 100 μl of lysis solution containing 20 mM Tris (pH 8), 137 mM NaCl, 1% Nonidet P-40, 10 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 1 mM sodium orthovanadatum (Na3VO4), 5 μg ml^{-1} leupeptin and 5 μg ml^{-1} aprotinin on ice for 45 min. The cell lysates were stored at −80°C until processing. Proteins were separated on 12% SDS-PAGE. The membranes were then washed three times in wash buffer and incubated overnight at 4°C with the specific primary Abs for IL-2Rγ (Santa Cruz, Santa Cruz, CA), caspase-3 (Cell Signaling, Danvers, MA), Bcl-2, Bcl-XL and beta-actin (Santa Cruz). Immune complexes were detected using the appropriate anti-rabbit or anti-mouse peroxidase-linked Abs. ECL kit (Amersham Biosciences, Brussels, Belgium) was used for visualization.

Densitometric analysis was performed after background equalization through the ImageJ software.

siRNAs and transfection

The validated chemically modified oligonucleotides used as short interfering RNA (siRNA) for IL2RG or random non-silencing nucleotides with no known specificity siRNA, used as negative control, were obtained from Invitrogen (Paisley, UK). These siRNAs were transfected at a concentration of 200 pmol/1×10^6 cells in a six well plate for 96 h. The transfection was performed by the lipid vector Lipofectamine 2000 kit (Invitrogen), according to the manufacturer's instructions. Preliminary experiments were performed to establish the silencing efficiency by testing two different oligonucleotides obtained from Invitrogen. The amount of protein expression reduction was calculated as follows: $1 - (OD_{siRNA} \times 100/OD_{control\ siRNA})$.

Real-time quantitative reverse transcriptase PCR analysis

Total RNAs were extracted using TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions. RNA was reverse transcribed in the presence of SuperScript II RT (Invitrogen) and oligo(dT) primers (Invitrogen) at 50°C for 50 min and then at 85°C for 5 min to inactivate the enzymes. Amplification of the cDNAs was performed using the SYBR Green and analyzed with the Light Cycler480 (Roche, Branchburg, NY). Primers are listed in Table 1. The PCR conditions comprised an initial denaturation at 94°C for 5 min, followed by 35 cycles at 62°C for 20 s and 72°C for 5 min. A dissociation procedure was performed to generate a melting curve for confirmation of amplification specificity. The results were normalized to beta-actin. The relative levels of gene expression are represented as $-\Delta Ct = (Ct_{gene} - Ct_{reference})$ and the fold change in gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method (where Ct is cycle threshold), as previously described (21).

Statistical analysis

MedCalc for Windows was used to analyze the data. The correlations between thymidine incorporation and γc expression and between cyclins and γc expression were obtained using the Pearson's correlation. $P < 0.05$ values were considered statistically significant.

Table 1. Primers used for real-time qRT-PCR

Gene	Primers sequence 5′-3′
Bcl-XL	5′-GTAAACTGGGGTCGCATTGT-3′ 5′-TGCTGCATTGTTCCCATAGA-3′
Cyclin D1	5′-AGGTCTGCGAGGAACAGAAGTG-3′ 5′-TGCAGGCGGCTCTTTTTC-3′
Cyclin D2	5′-CTGTGTGCCACCGACTTTAAGTT-3′ 5′-GATGGCTGCTCCCACACTTC-3′
Cyclin D3	5′-GCAGCGCCTTTCCCAACT-3′ 5′-TCAAAAGGAATGCTGGTGTATGTATC-3′
Cyclin A2	5′-CTGCTGCTATGCTGTTAGCC-3′ 5′-TGTTGGAGCAGCTAAGTCAAAA-3′
Cyclin B1	5′-CGGGAAGTCACTGGAAACAT-3′ 5′-AAACATGGCAGTGACACCAA-3′
IL-2Rγ	5′-TGCTAAAACTGCAGAATCTGGT-3′ 5′-AGCTGGGATTCACTCAGGTTTG-3′
Beta-actin	5′-GACAGGATGCAGAAGGAGAT-3′ 5′-GACAGGATGCAGAAGGAGAT-3′

Results

Deficiency in the expression of γc impairs cell survival

γc-dependent cytokines have important functions related to cellular survival (3). To determine whether γc deficiency had an effect on cell survival, we examined BCLs from healthy donors and X-SCID patients. The percentage of live cells was determined using trypan blue staining in the absence or presence of anti-Fas to trigger programmed cell death (22). In unstimulated X-SCID BCLs, there was an increase in the percentage of cell death, accounting for 25% as compared with 7% of control BCLs. Following 6 h of stimulation with anti-Fas, control and X-SCID BCLs showed a higher and comparable degree of cell death (32 and 36%) (Fig. 1A). Data on BCLs viability was confirmed by PI staining (data not shown). The increase of cell death observed is directly related to the lack of γc expression, since the neutralization of γc-dependent cytokines, as IL-2 or IL-4, did not modify the effect of γc on cell viability of normal cells (data not shown).

Programmed cell death is mainly mediated by activation of several caspases (23). These molecules exist as proforms that are activated by cleavage by the upstream caspase in the cascade (23). Western blot analysis with antibodies to the cleaved/activated form of caspase-3 revealed the presence of the cleaved protein only following anti-Fas stimulation (Fig. 1B), thus indicating that in unstimulated X-SCID BCLs the low viability was not a caspase-dependent process. Caspase-independent cell death has been attributed to mitochondrial damage (24), which can be regulated by Bcl-2 family members (25, 26). Bcl-2 and Bcl-XL operate as critical components in a complex network to integrate information and make ultimate life/death decisions (27). Since the γc-dependent cytokines promote cell survival by upregulating the antiapoptotic factor Bcl-2 (28) and Bcl-XL (29), the expression of Bcl-2 and Bcl-XL in control and X-SCID BCLs was evaluated. In γc-deficient cells, the expression of Bcl-2 and Bcl-XL was greatly decreased as compared with the control (Fig. 1B). These findings indicate that γc is required for cell survival and is dispensable for Fas-induced cell death. Moreover, the evaluation of molecular expression of Bcl-XL in unstimulated cells, through quantitative real-time

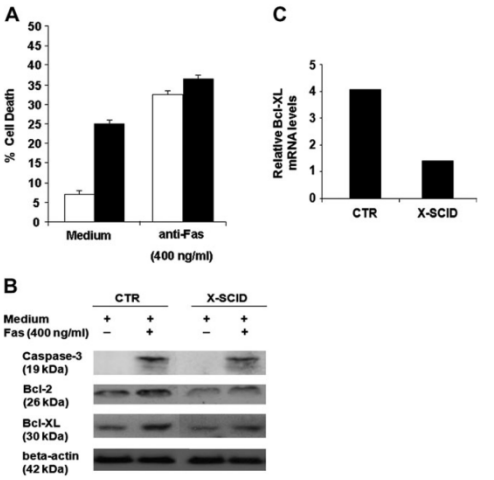

Fig. 1. Deficiency in the expression of γc has effect on cell survival. (A) BCLs were cultured in the absence or presence of 400 ng ml^{-1} anti-Fas for 6 h. The percentage of cell death was evaluated through trypan blue staining. Filled bars indicate BCLs from X-SCID patients; open bars BCLs from healthy donors (CTR). Data are expressed as mean (±SD) of six experiments. (B) BCLs were either cultured in medium alone or stimulated with anti-Fas. After 6 h, whole cell extracts were prepared, caspase-3, Bcl-2 and Bcl-XL expression was determined by western blotting. (C) mRNAs extracted from unstimulated cells were reverse transcribed and analyzed for the expression of Bcl-XL by qRT-PCR. Data were normalized to beta-actin.

PCR, revealed that in the X-SCID cells Bcl-XL mRNA was 35% of the control (Fig. 1C).

Correlation between γc expression and cell proliferation in malignant hematopoietic cell lines

Our previous studies demonstrated that γc exerts a role in cell cycle progression on BCLs in a concentration-dependent manner (11). To explore the potential oncogenic role of γc, we first examined the expression of the molecule in cell lines obtained from hematopoietic tumors, such as Molt-4, Raji, Rj225 and K-562. Synchronization of tumor cells in the G0/G1-phase of the cell cycle was accomplished by 12 h serum starvation. Western blot analysis revealed different γc expression levels among these cell lines. The protein was expressed predominantly in the K-562 and to a lesser extent in the Molt-4, Raji and Rj225 in a decreasing order. In X-SCID BCLs, γc expression was completely undetectable (Fig. 2A). Densitometric analysis is shown in the histogram in Fig. 2(B). The expression of γc was also evaluated in primary leukemic cell lines from three patients with ALL through quantitative real-time PCR. IL-2Rγ mRNA levels were increased in leukemic cells as compared with the control (Fig. 2C).

Moreover, to determine whether the expression levels of γc correlated with the self-sufficient growth in malignant cell lines, we examined the proliferation activity of cells under serum-deficient conditions. This was first evaluated by comparing the CFSE dilution profile, upon trypan blue exclusion

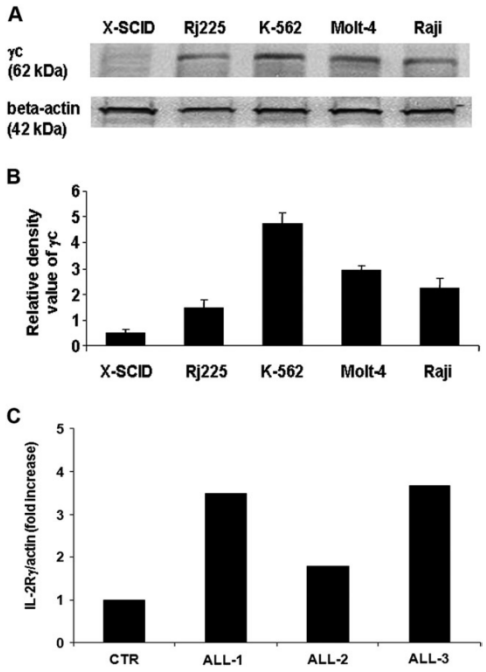

Fig. 2. Different γc expression levels in malignant cell lines. (A) X-SCID BCLs, Burkitt lymphoma cell line (Raji), the chronic myelogenous leukemia cell line (K-562), the human T-ALL cell line (Molt-4) and Raji isogenic derivative (Rj225) were lysed and immunoblotted for γc and beta-actin, as a loading control. (B) Densitometric analysis of the above western blot. ImageJ program was used to generate the data. Data are representative of six distinct experiments. (C) Primary leukemic cell lines, consisting of ALL cells, and control cells were analyzed for the γc expression by qRT-PCR. Histogram shows the relative gene expression as IL-2Rγ/actin fold increase.

assay, of malignant cells. After 5 h of serum-free culture, some variations in the rate of proliferation among the lines were already evident. As shown in Fig. 3(A and B), 20.19% of K-562 retained the dye, indicating a high proliferation rate, compared with 40.65% of Molt-4, 45.2% of Raji and 58.64% of Rj225. No proliferation was observed in X-SCID BCLs (Fig. 3A and B). CFSE experiments, at longer time points, were also performed in order to exclude a delayed entry into the cell cycle in the absence of γc. We found that longer time points were not discriminative as well, since neoplastic cells reach a plateau at 24–72 h of culture (data not shown). Moreover, the proliferation of these cell lines was also assessed by ^{3}H-thymidine incorporation assay after 4 days of culture. K-562 exhibited significantly higher thymidine incorporation (3851 ± 576 cpm) than Molt-4 (2224 ± 167 cpm), Raji (2167 ± 562 cpm) and Rj225 (1534 ± 115 cpm). As expected, there was no proliferation in X-SCID BCLs (Fig. 3C). These data were in keeping with the results of CFSE experiments. A statistically significant relationship between γc expression and spontaneous cell growth was documented in the examined cell lines (R = 0.98, P < 0.05) (Fig. 3D).

In the light of these findings, we hypothesized that the inhibition of γc expression in hematopoietic malignant cell lines might have a direct effect on proliferation of these cells. siRNA was used to knockdown the molecule in these cell lines. Efficiency and specificity of targeted siRNA sequences were confirmed by western blot analysis on total lysates and quantitative real-time PCR on mRNA. As shown in Fig. 4(A and B), the results of western blot assay revealed that at 96 h following the transfection, cells transduced with siRNA had less γc protein than the correspondent cells transduced with the control negative siRNA. In this representative experiment, γc-silencing reduced the amount of the protein in Rj225, K-562, Molt-4 and Raji by 80, 53, 62 and 32%, respectively. In addition, a decrease of the IL-2Rγ mRNA was observed in all cell lines, revealing a knockdown efficiency of ~85% in this experiment. In X-SCID cells, IL-2Rγ mRNA was undetectable (Fig. 4C). Moreover, as shown in Fig. 4(D), γc knockdown led to a significant decrease of proliferation. In particular, γc-silencing reduced cell proliferation of Rj225 by 40%, K-562 by 58%, Molt-4 by 45% and Raji by 50%, as compared with control siRNA cells (Fig. 4D). Taken together, these data confirm that γc plays a key role in the proliferation of these malignant cell lines.

Effect of γc on molecular mechanisms of cell cycle progression in malignant hematopoietic cell lines

To examine the mechanisms by which γc regulates cell cycle progression, we examined whether different amounts of the molecule were able to influence the transcription of genes selectively involved in cell cycle. Of note, cyclins are the key regulators of cell cycle progression (13). In particular, during the G0 to G1 phase transition, cyclins D1, D2 and D3 are the first molecules to be induced. Cyclin A2 gets activated during the transition from G1 to S phase and B type cyclins are detected during G2 exit and mitosis phase (30). In several kinds of malignant tumors, cyclins are overexpressed (31). Therefore, in all the above described cell lines, quantitative real-time analysis was performed to examine the effect of γc on the expression of cyclin A2, B1, D1, D2, D3 genes. As shown in Fig. 5(A), K-562 exhibited an up-regulation of D-type cyclins to a similar extent of Molt-4, while these genes were not expressed in Raji and X-SCID BCLs. In Rj225, only the cyclin D3 mRNA was up-regulated. Interestingly, we found that cyclins A2 and B1 were undetectable in X-SCID BCLs and reached a maximal expression in K-562 (Fig. 5B). In Molt-4, Rj225 and Raji, the levels of the cyclins A2 and B1 were lower than in K-562 (Fig. 5B). A direct correlation between cyclins A2 and B1 and γc expression was found (P < 0.05) (Fig. 5C and D). These data are consistent with the direct correlation previously shown between functional and molecular data of γc amount.

Discussion

Our results first indicate that in the absence of γc expression, BCLs die at a higher extent than control cells. This phenomenon is not related to abnormalities of regulatory

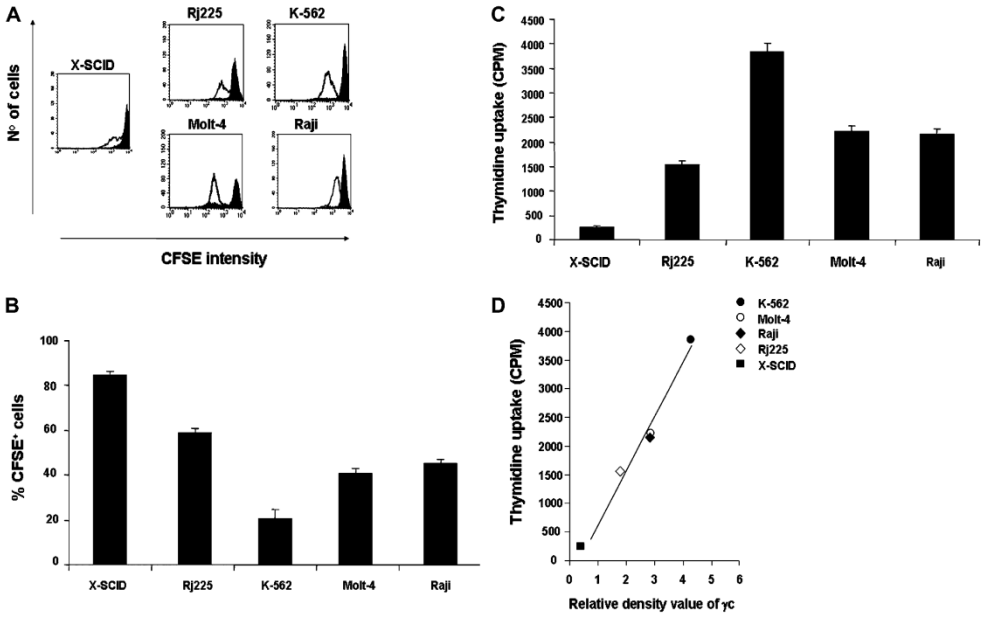

Fig. 3. Relationship between γc expression and spontaneous cell growth in malignant cell lines. (A) X-SCID BCLs, Raji, K-562, Molt-4 and Rj225 were cultured in the absence of serum and stained with 1.7 μM CFSE. After 6 h of culture, cells were analyzed by flow cytometry. Histograms show CFSE profiles 6 h following the start of culture. Solid black peaks represent the start of the culture. (B) Percentages of CFSE positive cells were obtained in the indicated cell lines by flow cytometry. Graphical representation of the mean (±SD) of percentage of CFSE positive cells for the three experiments conducted. (C) After starvation, X-SCID BCLs, Rj225, K-562, Molt-4 and Raji were cultured in serum-free medium for 4 days and pulsed with 0.5 μCi ^{3}H-thymidine for 8 h. Data represent mean (±SD) of six experiments. (D) Correlation between thymidine incorporation and γc expression in malignant cells. A positive correlation was demonstrated by the Pearson correlation coefficient ($R = 0.98$, $P < 0.05$).

mechanisms of apoptosis, in that, in non-stimulated cells, the absence of γc does not induce an increase of the activated form of caspase-3 that represents the central executioner molecule in the development of programmed cell death process. Hallmark of non-caspase-mediated cell death is the mitochondrial damage (24), which can be regulated by Bcl-2 family members (25). The γc-dependent cytokines promote cell survival by up-regulating the antiapoptotic Bcl-2 and Bcl-XL factors. In keeping with this, we found a decreased expression of Bcl-2 and Bcl-XL in γc-deficient cells. Of note, this effect was related to the lack of γc *per se* and not a secondary effect of unfunctional γc-dependent cytokines IL-2 or IL-4, in that neutralization of these cytokines did not modify cell viability of normal cells. As for the mechanism of non-caspase-mediated apoptosis, there is evidence indicating that autophagy may play an active role in cell death under unfavorable settings, such as nutrient or growth factors deprivation (27). However, whether γc is implicated in the autophagy process still needs to be addressed.

In spite of a wide number of studies on the molecular interactions and functions of γc, the precise role of this molecule in cell biology is still far from being clear. It has recently been documented a direct relationship between the amount of γc expression and the proliferative capability of control lymphoblastoid cells (11), implying a direct involvement of γc in self-sufficient growth in a concentration-dependent manner. Moreover, it was previously reported that *IL2RG* cooperates with *LMO2* in inducing hematopoietic tumors by studies of insertional mutagenesis in mice (32), thus giving a potential explanation to lymphoproliferative disorders occurring during gene therapy trials for X-SCID (9, 10). It is noteworthy that, differently from X-SCID, no clonal lymphoproliferation has been reported, to date, in patients receiving gene therapy for ADA deficiency (33), despite the observation of a similar frequency of integration sites near *LMO2* and other proto-oncogenes (34). Furthermore, a recent study, based on an experimental model of gene transfer in γc−/− mice, documented that γc overexpression could exert oncogenic properties by itself (35).

In this study, we observed that the amount of γc protein expression in several malignant cell lines directly correlates with spontaneous cell growth. We, also, found that the knockdown of γc molecule through siRNA is able to decrease the cell proliferation rate in these malignancies, thus confirming a direct involvement of the molecule as a key player in cell cycle progression.

The cancer is a multistep process that requires mutations of multiple molecules implicated in the biochemical signaling

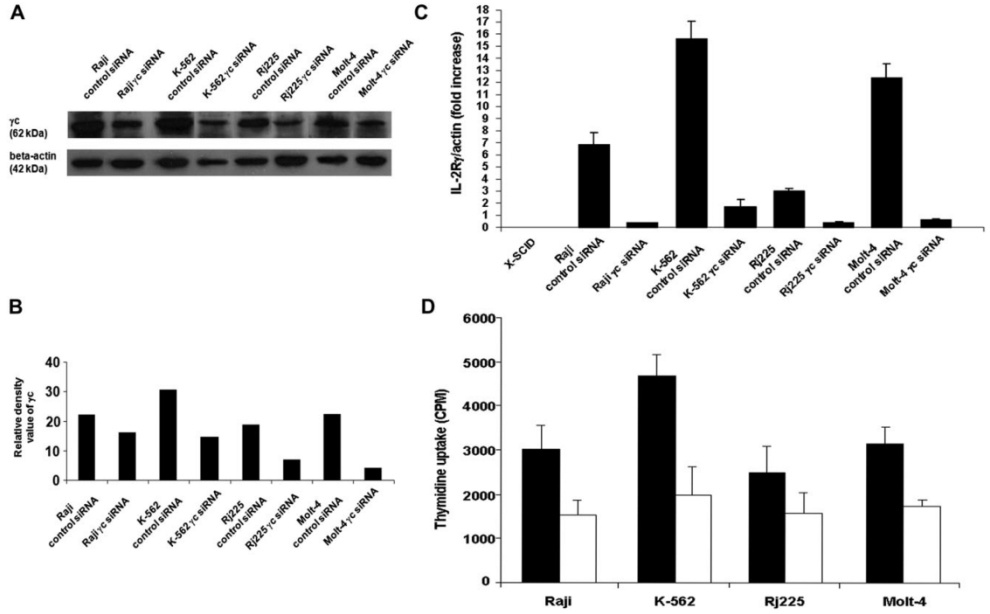

Fig. 4. Effect of γc siRNA transfection on the expression level of protein and cell proliferation of malignant cell lines. (A) Western blot analysis of γc and beta-actin protein expression in Rj225, K-562, Molt-4 and Raji transfected with γc siRNA or transfected with negative control siRNA after 96 h of transfection. (B) Densitometric analysis of the above western blot. ImageJ program was used to generate the data. Data were equalized for the background. Results are representative of five distinct experiments. (C) IL-2Rγ mRNA transcript evaluated by quantitative real-time PCR. Relative mRNA expression was determined using beta-actin control. (D) The proliferation of Rj225, K-562, Molt-4 and Raji transfected with γc siRNA or transfected with negative control siRNA was evaluated through ^{3}H-thymidine incorporation assay. Data represent mean (±SD) of five distinct experiments.

events of cell proliferation, thus providing growth advantage to the malignant cell (36). It is known that constitutive activation of JAK3 has been observed in a spectrum of lymphoid malignancies (37). In particular, JAK overexpression can be considered as one of the main biologic events leading to the constitutive activation of the JAK-STAT pathway that contributes to oncogenesis (38). It has been demonstrated that the amount of constitutively activated JAK3 parallels the extent of γc expression (11). Moreover, STAT molecules and, in particular the nearly identical STAT5 A and B (39), have been demonstrated to directly participate in tumor development and progression. STAT5 participates in oncogenesis through up-regulation of genes encoding cell cycle regulators, such as cyclins (40). Alterations in cell cycle machinery, mainly in the regulation of G1/S phase, are known to be associated to the development of solid tumors as well as hematological malignant diseases (31). In this context, a direct involvement of cyclins in malignant cell growth has been well documented and a correlation between the extent of cyclins expression/activation and the rate of proliferation has been found. Namely, cyclins A2 and B1 have been implicated in the pathogenesis of cancer and are overexpressed in several tumors (41, 42). Evidence indicates that these cyclins are key components of the cell-cycle machinery (43) and, in particular, cyclin A is expressed at high levels in hematopoietic stem cells and is essential for their proliferation (44). In our study, we observed that the expression of A2 and B1 cyclins strongly paralleled the proliferative capability of malignant cell lines. Interestingly, a positive correlation between the amount of γc and the expression of cyclins A2 and B1 was also found. Taken together, these data indicate that the higher is the rate proliferation of a certain cell line the higher is the expression of both γc and cyclins A2 and B1, thus confirming their involvement in the process in a concentration-dependent fashion.

We also found an increased expression of all D-type cyclins in those cell lines that proliferated mostly, K-562 and Molt-4, whereas they were not expressed in the other cell lines, but D3 found in Rj225. D-type cyclins are strongly expressed in many malignancies. Overexpression of cyclin D1 protein was documented in many forms of cancer, including breast cancer (45), while overexpression of cyclin D2 was noted in a wide range of B-cell malignancies, such as B-cell chronic lymphocytic leukemia (46). Like the other D cyclins, cyclin D3 is rearranged and the protein is overexpressed in several human lymphoid malignancies. It was documented that knockdown of cyclin D3 inhibits the proliferation of ALL cells (47). However, while A- and B-type cyclins seem to be vital and necessary components of cell cycle progression (44), D-type cyclins may be dispensable

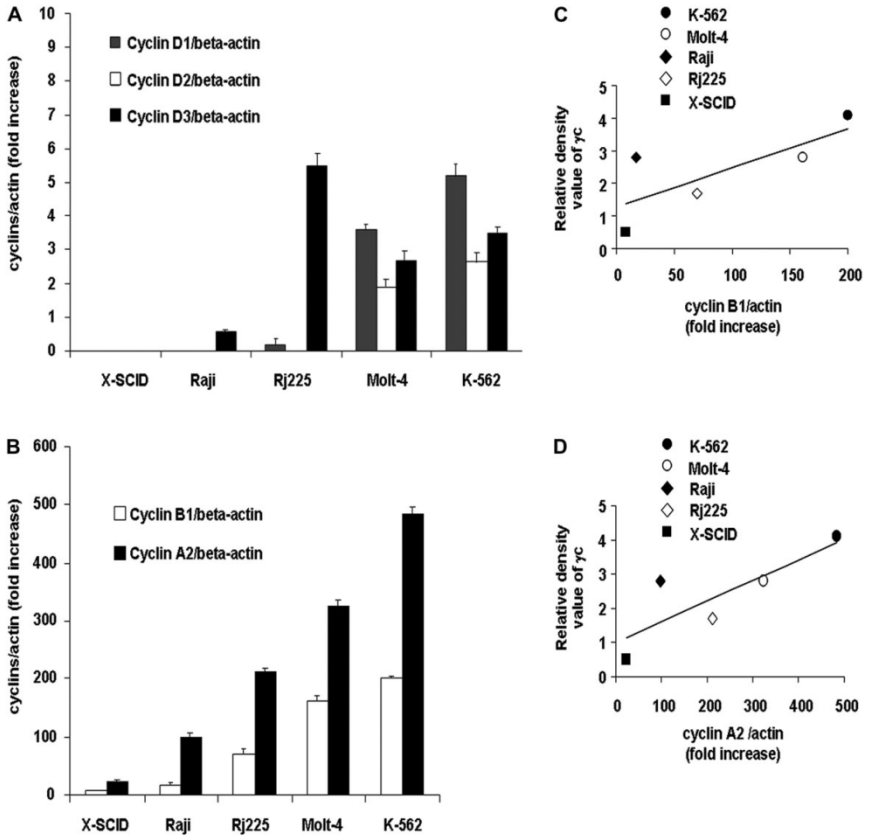

Fig. 5. Cyclins expression is up-regulated in malignant cell lines. (A–B) RNAs extracted and reverse transcribed were analyzed for the expression of D1, D2, D3 and A2, B1 cyclins by qRT-PCR. Histograms show the relative gene expression as cyclin/actin fold increase. Relative expression of cyclins were calculated for each cell line after normalizing against beta-actin. (C) Correlation between γc protein amount, expressed as relative density, and fold increase cyclin B1/actin expression. (D) Correlation between γc protein amount, expressed as relative density, and fold increase cyclin A2/actin expression.

for proliferation under certain circumstances, in that different cell types are sensitive to cyclin D knockdown at a different extent (48). This would suggest that they regulate cell cycle in a cell type-specific manner and that there are alternative mechanisms allowing cell cycle progression in a cyclin D-independent fashion (48). Anyway, a critical role for oncogenic transformation of D-type cyclins is a well-established feature.

Our data indicate that γc is strongly implicated in cell cycle progression of hematopoietic malignancies in a similar fashion to the role played in control lymphoblastoid cells, as previously shown. This biologic effect is strictly dependent on the expression level of the molecule and can be abolished by gene knockdown. Of note, a direct correlation between the amount of γc expression and the proliferative capability of the malignant cell lines and the regulatory elements of cell cycle progression, A and B cyclins, was found. Moreover, we documented that the IL-2Rγ mRNA was also highly expressed in primary leukemic cells, thus confirming a direct involvement of the γc in tumor growth. Our data could provide the basis to develop in the near future new therapeutic strategies targeting this molecule in cancer therapy. Moreover, this information may also help understand undesired side effects of gene therapy trials.

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§ 3.2 ATM and Hyper IgM: cancer predisposition and neurodegeneration

As previously mentioned, the genetic defect underlying the immunodeficiency may play a direct role in cancer pathogenesis through genetic mutations in DNA DSBs repair systems, which lead to accumulation of mutations that promote tumorogenesis. Indeed, an high cancer susceptibility has been reported for ataxia-telangiectasia (A-T) [196]. The disease is associated with mutations in the ataxia telangiectasia mutated (ATM) gene encoding for a 370 kDa serine/threonine kinase. The features of A-T include telangiectasia, immune and endocrine dysfunctions, cellular radiosensitivity, genomic instability, premature aging, and predisposition to cancer [197,198]. The prevalence of cancer in A-T is 10-30%, representing the second cause of death [141,199]. Leukemia and lymphoma account for about 85% of malignancies [141]. Other solid tumors including ovarian cancer, breast cancer, gastric cancer, melanoma and gonadic cancer have been described. Patients with A-T also show an increased sensitivity to ionizing radiations. The hallmarks of the disease are related to the progressive neurological dysfunction, especially affecting the cerebellum and resulting in uncoordinated and ataxic movements associated to a deterioration of gross and fine motor skills. Recently, an improvement of neurological signs during short-term treatment with betamethasone has been reported. To date, the molecular and biochemical mechanisms by which the steroid produces such effects have not yet been elucidated.

Other forms of immunodeficiency are associated to cancer predisposition, such as the Hyper-IgM (HIGM) syndromes, caused by mutations in the genes encoding for the CD40/CD40L interaction pathway leading to defects in the class switch recombination. X-linked hyper-IgM syndrome is associated with a highest risk to develop carcinomas of the liver, pancreas, biliary tract and associated neuroectodermal endocrine cells [142].

HIGM is a heterogeneous group of immune defects characterized by normal or increased production of IgM contrasting with a marked decrease or an absence of other isotypes. Patients with HIGM often present infections by opportunistic

intracellular pathogens. Significant neurologic complications, such as cerebral toxoplasmosis [200,201] and cryptococcosis [202], are seen in 10-to- 15% of affected males. Malignancies may also occur in patients with HIGM and usually affect the biliary tree [143,203] and the gastrointestinal tract in the form of neuroendocrine tumours [204]. As in other immunodeficiencies, patients also have an increased risk for lymphomas, particularly Hodgkin's disease associated with Epstein-Barr virus infection [198]. Furthermore, lymphomas are common in forms of HIGM due to DNA repair defects such as Ataxia-telangiectasia and Nijmegen Breakage syndrome.

We reported the case of a child, in whom a clinical and functional diagnosis of Hyper-IgM syndromes was achieved, with a cutaneous B-cells lymphoma developed at the age of 12 years. The patient described presented with recurrent upper and lower respiratory infections and evidence of suppurative lung disease at the conventional chest imaging. The presence of low serum IgG and IgA levels, elevated IgM levels, and a marked reduction of in vivo switched memory B cells led to a clinical and functional diagnosis of HIGM although the genetic cause was not identified.

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REVIEW ARTICLE

Betamethasone therapy in Ataxia Telangiectasia: unraveling the rationale of this serendipitous observation on the basis of the pathogenesis

G. Giardino, A. Fusco, R. Romano, V. Gallo, F. Maio, T. Esposito, L. Palamaro, G. Parenti, M. C. Salerno, P. Vajro and C. Pignata

Department of Pediatrics, Federico II University, Naples, Italy

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Ataxia telangiectasia (A-T) is a rare autosomal recessive disorder characterized by progressive neurological dysfunction. To date, only supportive care aimed to halt the progressive neurodegeneration is available for the treatment. Recently, an improvement of neurological signs during short-term treatment with betamethasone has been reported. To date, the molecular and biochemical mechanisms by which the steroid produces such effects have not yet been elucidated. Therefore, a review of the literature was carried out to define the potential molecular and functional targets of the steroid effects in A-T. Glucocorticoids (GCs) are capable to diffusing into the CNS by crossing the blood–brain barrier (BBB) where they exert effects on the suppression of inflammation or as antioxidant. GCs have been shown to protect post-mitotic neurons from apoptosis. Eventually, GCs may also modulate synaptic plasticity. A better understanding of the mechanisms of action of GCs in the brain is needed, because in A-T during the initial phase of cell loss, the neurological impairment may be rescued by interfering the biochemical pathways. This would open a new window of intervention in this so far non-curable disease.

Introduction

Ataxia telangiectasia (A-T) is a rare autosomal recessive disorder that affects several body systems and tissues leading to a complex and severe phenotype [1,2]. The hallmark of A-T is the progressive neurological dysfunction characterized by uncoordinated and ataxic movements as a result of cerebellar atrophy or dysfunction [3]. Other features of A-T include telangiectasia, immune and endocrine dysfunctions, cellular radiosensitivity, genomic instability, premature aging, and predisposition to cancer [3,4]. The prognosis for survival is poor, and death usually occurs during about the second or third decade of life predominantly caused by the progressive neurodegeneration, pulmonary failure with or without identifiable pneumonia, or cancer [5]. Currently, there is no effective treatment for A-T, but only supportive care aimed to halt progressive neurodegenerative changes. In a recent study, we reported on the amelioration of neurological signs, assessed by Scale for the Assessment and Rating of Ataxia (SARA), during short-term treatment with oral betamethasone [6,7]. Far from being a definitive therapeutic approach, this observation makes us able to argue that, during the initial phase of cell loss, biochemical and functional cerebellar abnormalities may still be modified, by interfering the biochemical pathways.

As for the intimate mechanism implicated in such beneficial effect of the betamethasone, no conclusive information is available. The central issue is to clarify whether this improvement is related to the peripheral effect of the drug or rather to an effect of the drug on the central nervous system (CNS) performance activity. On peripheral blood mononuclear cells, an effect of betamethasone on radical oxygen species (ROS) production has been documented [8], and evidence indicates that steroids may cross the blood–brain barrier (BBB). In this review, we will focus on new aspects of pathophysiology and their implication in understanding the mechanism of action of betamethasone in A-T.

ATM defect and A-T pathogenesis

A-T is associated with mutations in the ATM gene encoding for a 370-kDa serine/threonine kinase, which

Correspondence: C. Pignata, Professor of Pediatrics, Department of Pediatrics, Unit of Immunology, Federico II University, via S. Pansini, 5-80131 Naples, Italy (tel.: +39 081 7464340; fax: +39 081 5451278; e-mail: pignata@unina.it).

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shares sequence similarities with the catalytic subunit of phosphatidyl inositol-3 kinase. The ATM gene is large, spanning 150 kb of genomic DNA and encoding a ubiquitously expressed transcript of approximately 13 kb [9]. The classic form of A-T results from the presence of two truncating ATM mutations, leading to total loss of function of the ATM protein, whereas milder forms are associated with a leaky splice site ATM mutation or with mis-sense mutations. The presence of these latter alterations may allow some expression of mutant ATM with a degree of residual kinase activity [10].

ATM plays a key role in the control of various cellular processes such as DNA repair, cell cycle progression, gene transcription, protein synthesis and degradation, and apoptosis (Fig. 1). The disease may be considered a prototype of the DNA repair defect syndromes. In fact, ATM represents the central component of the signal transduction pathway responding to double-strand DNA breaks (DSBs) caused by IR, endogenous and exogenous DNA-damaging agents (Fig. 1) [11]. A-T demonstrates the typical consequences of defects in the DNA damage reponse (DDR): degeneration of specific tissue affecting particularly the nervous and immune systems, chromosomal instability, and sensitivity to specific DNA-damaging agents. However, the neuronal degeneration in A-T is only partially due to defective DNA damage response DDR [12] and recognizes a complex pathogenesis. It has been recently proposed that ATM also acts as a redox sensor. ATM activation may derive directly from oxidative stress or DNA damage induced by oxidative stress. In the first case, a disulfide bridge is formed between cysteine (C2991) residues in an ATM dimer [13], whilst in the second case an active monomer forms from an inactive dimer [14]. This evidence suggests a role of ATM in signaling other than direct DNA damage. Furthermore, recent studies highlight the role of ATM in modulating mitochondrial homeostasis. A fraction of ATM localizes to the mitochondria and can be activated by mitochondrial dysfunction in the absence of DNA damage [15]. The high rate of transcription and translation in neurons creates a stressful environment, where neuronal genomic and mitochondrial DNA are constantly attacked. Mitochondrial dysfunction has been found to be implicated in a significant number of neurological diseases, including Parkinson's disease, Huntington's

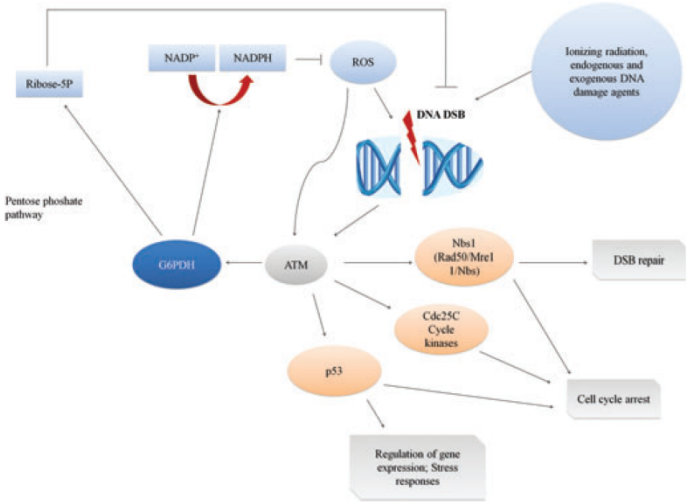

Figure 1 Following the induction of double-strand DNA breaks (DSB), A-T mutated (ATM) is activated, and a portion of nuclear ATM binds to the DSB sites. Activated ATM is then able to phosphorylate substrates that are involved in a series of events including cell cycle checkpoint activation and apoptosis. ATM-dependent phosphorylation of p53 and Chk2 can inhibit the cell cycle at various phases. ATM also phosphorylates NBS1, which is involved in the intra-S-phase checkpoint. ATM plays a key role also in maintaining the reducing power of the cellular environment upregulating pentose phosphate pathway (PPP), which converts glucose-6-phosphate to ribose-5-phosphate. In this process, glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) reduce NADP to NADPH. High levels of ROS, under normal conditions, activate ATM, which, in turn, promotes G6PD activity, thus restoring the redox state of the cells. Oxidative stress contributes to the accumulation of DNA damage, leading to ATM activation. However, ATM oxidation directly induces its activation in the absence of DSBs.

disease, Alzheimer's disease, amyotrophic lateral sclerosis, and various peripheral neuropathies, as well as the normal aging process [16,17]. These findings suggest that the mitochondrial dysfunction and the increase in mitochondrial reactive oxygen species may contribute to the A-T phenotype.

DNA repair defect, and neurodegeneration

A-T provides a well-characterized example of the relationships that exist between repair defects and neurodegenerative disease.

The high rates of transcription and translation in neurons are associated with high rates of metabolism and mitochondrial activity. The amount of oxygen consumed by the brain relative to its size far exceeds that of other organs. This high activity creates a stressful environment where neuronal genomic and mitochondrial DNA are constantly attacked by damaging metabolic by-products, primarily ROS [18]. Because neurons are post-mitotic cells and, in case they are irreversibly damaged, they cannot be replaced, they should survive as long as the organism does. Thus, they have elaborated, stringent defense mechanisms to ensure their longevity [19], such as DDR, an elaborate signaling network activated by DNA damage [20,21]. Genetic deficiencies in enzymes that detect or repair DNA damage can induce the apoptosis of specific neuronal populations or further sensitize them to genotoxic stresses [22]. Accumulation of DNA damage in neurons is one of the major forms of damage involved in brain aging and neurodegeneration. Many neurodegenerative syndromes result from defective DNA strand break responses. However, the disease-specific hallmarks of A-T with regard to the onset and course of neurodegeneration likely reflect selective DDR requirements in the specific areas of CNS [23]. ATM signaling functions predominantly in recently post-mitotic neurons [24] to trigger apoptosis of cells, which have experienced excess DNA damage during brain development. A failure to do this may result in the establishment of genetic lesions that eventually result in cell loss and neurodegeneration [25]. According to this model, in the absence of ATM, damaged neurons survive and populate the Purkinje neuron layer, only to degenerate later as a result of DNA damage experienced during their development.

Autoptical and bioptical studies show atrophy of the cerebellar folia, widespread loss of Purkinje cells, granule cell loss, and significant thinning of the molecular layer of the cerebellum. Purkinje cells have abnormally smooth dendrites with reduced arborizations, and they are often displaced in the molecular layer of the cerebellum [26]. Because basket cell interneurons are present in the cerebellum, it is likely that the Purkinje cell layer, initially formed, is later followed by progressive degeneration. Even though the neurodegenerative phenotype in younger A-T patients is restricted to Purkinje and granule cells of the cerebellum, it broadens considerably with age and may extend to the basal ganglia. This widespread degeneration results in a progressive decline in neural function [23].

ATM and oxidative stress

ATM appears to be involved in the response to oxidative stress [27], possibly acting as a sensor of ROS [13,28]. The generation of ROS can arise either from toxic insults or from normal metabolic processes. The overproduction of oxidants and/or dysfunction of endogenous antioxidant defenses result in oxidative stress–induced injury with damage to all the major classes of biological macromolecules, such as nucleic acids, proteins, lipids, and carbohydrates [29]. In mammalian cells, several defense mechanisms against oxidative damage have been documented [30,31]. The first one is based on low molecular weight antioxidants or protein scavengers [32] such as glutathione, which appears in both its oxidized (GSSG) and reduced (GSH) forms [32]. The second one includes antioxidant enzymes like superoxide dismutases, SOD1 and SOD2, glutathione peroxidase, catalase [29], and cytochrome p450 reductase [33].

The CNS is particularly vulnerable to oxidative stress due to the high rate of metabolism, the disproportionately low levels of oxidative defense mechanisms, and the high content of easily oxidized substrates. In this context, an increased and unopposed ROS production can lead to neurotoxicity resulting in neural damage and eventually cell death [29]. Evidences support the direct relationship between excessive ROS production and the pathogenesis of A-T [34]. $ATM^{-/-}$ cells exhibit high concentrations of ROS and hypersensitivity to agents that induce oxidative stress [32], and A-T lymphoblasts reduce glutathione more slowly than normal cells after glutathione depletion induced by oxidative stress [35]. However, other works failed to identify an impaired GSH biosynthesis in cultured A-T cells [36,37]. Moreover, recent studies have documented the presence of high levels of oxidative damage in patients with A-T [8,38], confirming previous observations in mouse models [39]. We also observed in a group of patients with A-T the existence of an inverse correlation between the degree of cerebellar atrophy and GSH levels [8]. Furthermore, in ATM-deficient mice, the overexpression of SOD1 exacerbated certain features of the A-T

phenotype [40]. These observations suggest that the impaired response to ROS in A-T cells might influence neuronal survival [41].

ATM deficiency causes oxidative damage to proteins and lipids in brain, testes, and thymus, genomic instability and hypersensitivity to IR and other treatments that generate ROS [34]. A recent study shows that ATM oxidation directly induces its activation in the absence of DSBs [42], confirming that ATM acts as sensor of ROS in human cells [42].

ATM plays a key role in maintaining the reducing power of the cellular environment upregulating pentose phosphate pathway (PPP), which converts glucose-6-phosphate to ribose-5-phosphate [41]. In this process, glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) reduce NADP to NADPH [43]. High levels of ROS, under normal conditions, activate ATM, which, in turn, promotes G6PD activity, thus restoring the redox state of the cells. In patients with A-T, this feedback might be compromised, leading to the accumulation of ROS [41]. Similar to ATM-deficient cells, G6PD-deficient cells are more sensitive to apoptosis induced by IR [44].

It has been observed that ATM activation increases G6PD favoring the interaction between heat shock protein (HSP) 27 and G6PD [41]. ATM in fact induces serine 78 phosphorylation of HSP27 and mediates G6PD activation. It is possible that this phosphorylation increases HSP27 affinity for G6PD increasing, in turn, its activity.

A-T and steroid therapy

Currently, there is no effective treatment for A-T. A single case report pointed out that steroids produced in a child a short-term improvement in ataxia [45]. Recent clinical reports extended this clinical observation and documented a clear-cut beneficial effect of such therapy that was inversely correlated with the extent of cerebellar atrophy. This beneficial effect was also inversely correlated with the age of the patients [8]. Of note, this effect was strictly drug dependent, in that the drug withdrawal paralleled the worsening of the neurological signs [6]. A recent double-blind, randomized, placebo-controlled crossover trial has confirmed the previous observations [46]. Intriguingly, during the short steroid trial, a paradoxical effect on the proliferative response to mitogen stimulation was documented, differently to what expected on the basis of the drug-induced immune suppression [7]. This finding would potentially imply a direct effect of betamethasone on the intimate altered pathogenetic mechanism in A-T [7].

However, as for the intimate mechanism implicated in such beneficial effect of the betamethasone, no conclusive information is available.

Peripheral effects of glucocorticoids

Most of the biologic effects of glucocorticoids (GCs) have been studied in white blood cells and, in particular, in lymphocytes. Different mechanisms of action have been described to explain GC therapeutic effects: the classical genomic mechanism of action mediated by the GC/GC receptor (GR) complex and non-genomic effects mediated by the cytoplasmic or membrane-bound GC/GR complex. The genomic mechanism may result in transrepression or transactivation [47] (Fig. 2). As for the transrepression (Fig. 2), GC/GR complex directly or indirectly interacts with transcription factors, including NF-KB, NF-AT, and AP-1 [48], thus resulting in the down-regulation of well-defined target genes [49]. Through this mechanism, GCs inhibit the synthesis of T-helper type 1 cytokines and, in particular, interleukin (IL)-2 [50], thus explaining the potent anti-inflammatory properties of these drugs. GCs also act, to a lesser extent, on the down-regulation of chemokine secretion and the expression of costimulatory molecules from immune and endothelial cells [51].

GCs seem to act downstream the TCR signal transduction pathway and, in particular, on the calcium signaling induced by inositol 1,4,5-triphosphate (IP3) [52]. It has been shown that low doses of dexamethasone on immature T cells (1–10 nM) induce the inhibition of Lck, thus altering calcium flux and the expression of IP3 receptor. In conclusion, these events eventually lead to the inhibition of the TCR-mediated signaling, thus explaining the inhibitory effect on cell activation [52]. By contrast, GCs seem to exert moderate effects on the survival, proliferation, and functionality of B cells [51].

Differently to this process, through a transactivation mechanism (Fig. 2), GC/GR complex directly interacts with molecular targets as GC-regulated genes. Following the interaction of GCs with GR, GRs migrate into the nucleus and, subsequently, bind to GC-responsive element (GRE), thus modulating the transcription of a variety of genes including GC-induced leucine zipper (GILZ) and GC-induced TNFR family-related (GITR) [53,54], which are the major biomarkers of GC-induced gene transcription. GILZ is a 137-amino-acid leucine zipper protein rapidly induced by GCs implicated in the modulation of activation-induced cell death [54,55], through inhibition of NF-kB translocation and activation. It is also able to regulate T lymphocyte activity, including T-cell survival [55,56].

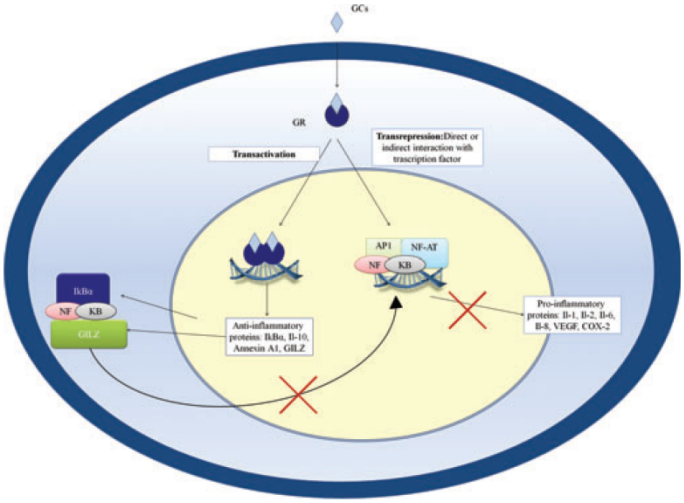

Figure 2 The classical, genomic mechanism of glucocorticoid action can be divided into two processes: 'transrepression' and 'transactivation'. Transrepression is the molecular mechanism by which GC/GR complex directly or indirectly interacts with transcription factors, including NF-KB, NF-AT, and AP-1, thus resulting in the down-regulation of well-defined target genes. In particular, through this mechanism, GCs inhibit the synthesis of T-helper type 1 cytokine. Transactivation is referred to the molecular mechanism by which GC/GR complex directly interacts with GC-regulated genes. GCs bind and induce GR activation, followed by the GR translocation to nucleus and subsequent binding to GC-responsive element (GRE), thus modulating the transcription of a variety of genes including GC-induced leucine zipper (GILZ) and GC-induced TNFR family–related (GITR) molecules, which are biomarkers of the GC-induced gene transcription.

GC effects on CNS

Neuroprotection

In the brain, the biologic function of GCs relies on their ability to access brain targets [57]. Data are available indicating that cortisol and corticosterone are capable of diffusing from plasma into the CNS by crossing the BBB and blood–cerebrospinal fluid barrier (BCSFB) [57]. However, the anatomical complexity of the brain enormously complicates the studies in the brain aimed at defining in detail gene targets of GC/GR complex [58]. Receptor binding and immunocytochemistry studies reveal that different regions of CNS do express GR, although the pattern of expression varies amongst the areas. GCs have been documented to exert neuroprotective, anticonvulsive, and anxiolytic properties [59]. GCs also have effects on the behavior mediated by non-genomic mechanisms [60]. Under physiological conditions, moderate increases in GCs have been shown to modulate cognition patterns, as well [59,61].

In several neurodegenerative disorders, inappropriate apoptosis seems to play an important pathogenic role. GCs exhibit a prominent role in the regulation of apoptosis, even though the modulatory effect occurs through opposite mechanisms under different circumstances. GCs enhance apoptosis in inflammatory and immune cells, whereas they seem to protect tissues in which the inflammation takes place from apoptotic death [62,63]. GCs promote apoptosis by inducing proteasome activity. GCs have been shown to protect neurons from apoptosis through a mechanism involving the cyclin-dependent kinase inhibitor p21Waf1/Cip1 molecule [62]. p21Waf1/Cip1 is upregulated and phosphorylated by GCs under the control of PI3-Akt kinase, eventually leading to the activation and cytoplasmic translocation of p21Waf1/Cip1 [62]. This modulatory pathway represents a novel antiapoptotic mechanism, which, in particular, exerts its effects on post-mitotic neurons, thus implying a direct role of p21Waf1/Cip1 as a molecular target of GC-induced neuroprotection [62]. Caspase-3 participates in the process, as well, being inhibited by p21Waf1/Cip1 [62].

GCs also act on neurological functions by upregulating HSP27 in the cerebellum [64]. This molecule belongs to the family of HSPs, which are small molecular weight proteins acting as molecular chaperones involved in the regulation of actin polymerization, thermotolerance, and cell growth [65]. HSPs have a protective function after exposure to several cellular insults, such as ROS. Synthesis of such proteins is

induced by GCs [64], and the resistance of the brain to stress-like elevations in corticosterone levels seems mediated by this mechanism [64]. The cerebellum is the principal site of induction of HSPs, and in particular HSP27, which seems to have a unique role in adapting neurons [66].

In conclusion, GCs act as inducers of protective systems against several insults in the brain. Several lines of evidence support the hypothesis that such mechanisms might have some site-directed specificity in the brain.

Synaptic plasticity

Plasticity refers to the ability of the CNS to reorganize itself over the time [67]. The main mechanism includes functional changes or alterations in the number or location of synapses between neurons [68]. Under physiological conditions, moderate increases in GCs have been shown to modulate synaptic plasticity and cognition patterns [69]. GRs exert their effects at the membrane, where they modulate the conductance of specific ion channels, which, in turn, modulate neuronal excitability [70]. GC injections in mice cause a dose-dependent effect on dendritic spine dynamics, increasing spine turnover within brain cortex [71]. Accordingly, a reduction in endogenous GC activity causes a substantial reduction in spine turnover rates [71]. However, chronic GC excess leads to abnormal loss of stable connections that were established early in life [71].

Synaptic plasticity requires brain-derived neurotrophic factor (BDNF) secretion and TrkB activation [72]. Trk receptors are involved in neuronal survival and differentiation [73,74]. Acute administration of dexamethasone promotes TrkB activation in the CNS *in vivo*, the effect not being mediated by BDNF. The activation of Trk receptors by GCs requires a genomic action of GR [75]. Also in the neurons, in addition to the genomic mechanism, GRs may also act in extranuclear areas, as the dendritic spine and axon terminal to induce the spinogenesis, or to modulate locally the synaptic transmission.

In a recent study, through functional magnetic imaging, an increase in the activation in relevant cortical areas has been shown to be coupled to changes in the motor performance in A-T patients treated with betamethasone [76]. This observation suggests that in patients with A-T, steroid treatment could improve motor performance facilitating cortical compensatory mechanisms. Changes in clusters of activation in response to motor tasks or sensory stimuli, related to the improvement in motor performance, have also been described during the rehabilitation of patients with different brain injuries, such as focal resection of the cortex or hemispherectomy [77] or stroke [68]. This ability to promote brain remodeling, reducing the impact of disability, has been related to the neuronal plasticity [68].

Conclusion

Currently, there is no effective treatment for A-T. Recent studies reported on the improvement of neurological signs during short-term treatment with oral betamethasone [6,7,45]. As for the mechanism underlying this effect of corticosteroids on neurological symptoms in A-T, no definitive explanation is currently available.

In this review, a possible mechanism implicated in the beneficial effect of steroids has been discussed on the basis of the pathogenesis, in particular, the GC effects on the suppression of inflammation or as an antioxidant through the activation of several biochemical pathways.

In several neurodegenerative disorders, and in particular in A-T, an inappropriate apoptosis seems to play an important pathogenic role. Studies show that GCs are able to enhance apoptosis in inflammatory and immune cells, whereas, at the same, time they seem to protect tissues in which the inflammation takes place [62,63]. GCs have been shown to protect post-mitotic neurons from apoptosis through a mechanism involving the cyclin-dependent kinase inhibitor p21Waf1/Cip1 molecule [62]. Of note, ATM signaling appears to function predominantly in immature, recently post-mitotic neurons [24].

Even though steroids are not curative and cannot be proposed for long-term therapies due to the side effects, it is noteworthy that betamethasone studies highlighted that during the clinical course of the disease, there is a phase when neurological impairment may be rescued at some extent. This observation will open novel therapeutic strategies.

Disclosure of conflict of interest

The authors declare no financial or other conflict of interests.

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CASE REPORT Open Access

Hyper IgM syndrome presenting as chronic suppurative lung disease

Silvia Montella[1], Marco Maglione[1], Giuliana Giardino[1], Angela Di Giorgio[1], Loredana Palamaro[1], Virginia Mirra[1], Matilde Valeria Ursini[2], Mariacarolina Salerno[1], Claudio Pignata[1], Carlo Caffarelli[3] and Francesca Santamaria[1*]

Abstract

The Hyper-immunoglobulin M syndromes (HIGM) are a heterogeneous group of genetic disorders resulting in defects of immunoglobulin class switch recombination. Affected patients show humoral immunodeficiency and high susceptibility to opportunistic infections. Elevated serum IgM levels are the hallmark of the disease, even though in few rare cases they may be in the normal range. Hyper IgM is associated with low to undetectable levels of serum IgG, IgA, and IgE. In some cases, alterations in different genes may be identified. Mutations in five genes have so far been associated to the disease, which can be inherited with an X-linked (CD40 ligand, and nuclear factor-kB essential modulator defects) or an autosomal recessive (CD40, activation-induced cytidine deaminase, and uracil-DNA glycosylase mutation) pattern. The patient herein described presented with recurrent upper and lower respiratory infections and evidence of suppurative lung disease at the conventional chest imaging. The presence of low serum IgG and IgA levels, elevated IgM levels, and a marked reduction of in vivo switched memory B cells led to a clinical and functional diagnosis of HIGM although the genetic cause was not identified.

Background

Chronic suppurative lung disease (CSLD) describes a clinical syndrome characterized by chronic endobronchial suppuration with or without high resolution computed tomography (HRCT) evidence of bronchiectasis [1,2]. The presenting symptoms are identical to bronchiectasis, and include recurrent chest infections with prolonged moist or productive cough, exertional dyspnoea, features of reactive airway disease, and in some cases growth failure. Digital clubbing, chest wall deformity, adventitious sounds, and/or hyperinflation are the main physical signs. Haemoptysis is rare in children. Once excluded cystic fibrosis (CF), patients should be investigated for other disorders, such as primary immunodeficiencies, aspiration pneumonia, and primary ciliary dyskinesia (PCD) [3].

Hyper-immunoglobulin M syndrome (HIGM) is a rare (incidence, 1 in 100,000 births) primary immunodeficiency [4,5], in which defective B cell isotype switching leads to a phenotype characterized by elevated or normal levels of serum IgM, and low levels of serum IgG, IgA and IgE [5]. Mutations in five different genes, encoding for CD40 ligand, CD40, nuclear factor-kB essential modulator (NEMO), activation-induced cytidine deaminase (AID), and uracil-DNA glycosylase (UNG), have so far been associated to the disease [6-8]. Like in other humoral immunodeficiencies, recurrent respiratory tract infections, potentially leading to bronchiectasis, sinus infections, and ear infections are commonly found in affected patients. Immunoglobulin replacement therapy prevents the progression of the clinical manifestations. In around 40% of cases opportunistic infections by *Pneumocystis carinii* is the presenting feature of the syndrome [9,10]. However, despite the high susceptibility to airway infections, lung involvement is not commonly characterized by CSLD.

We herein describe the case of a 7-year old girl presenting with symptoms and signs of CSLD, in whom a functional and clinical diagnosis of HIGM was achieved.

Case presentation

A 7-year old girl complaining of recurrent upper and lower respiratory infections and chronic productive cough was referred to our Department for further diagnostic work-up. She was born to non-consanguineous

* Correspondence: santamar@unina.it
[1]Department of Pediatrics, Federico II University, via Pansini 5, Naples 80131, Italy
Full list of author information is available at the end of the article

parents after an uneventful 37 weeks pregnancy and had been healthy until the age of three years, when recurrent upper respiratory tract infections started. No other problems were experienced by the patient until the age of 6 years, when she developed an acute pneumonia during a measles infection. Since then, recurrent lower airway infections requiring repeated antibiotic courses and several hospital admissions occurred.

At admission to our Department, the patient appeared in good clinical conditions and physical respiratory examination disclosed any remarkable sign except for mild rhinorrhea and productive cough. Lung auscultation revealed diffuse mild crackles and rhonchi. No symptoms and signs of heart disease were observed. Lung function tests showed no relevant impairment, with forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC) of 106% and 104% predicted, respectively. Bronchiectasis in the left and right lower lobes and a consolidation area in the middle lobe were evident at chest HRCT (Figure 1). Tuberculosis, CF, PCD, gastroesophageal reflux disease, alpha-1 anti-trypsin deficiency and atopy were ruled out on the basis of normal or negative results of purified protein derivative test, sweat test, cilia motility and ultrastructure assessment at nasal brushing, prolonged pH-metry, serum alpha-1 anti-trypsin level, and skin prick test and serum IgE levels to the most common food and inhalant allergens. General blood test results were unremarkable, but raised levels of serum IgM (5.63 g/l; normal range, 0.56-2.61) associated with low serum concentration of IgG and IgA (6.06 g/l; normal age-matched range, 6.33-10.16; and 0.33 g/l; normal range, 0.41-3.15, respectively) suggested the diagnosis of HIGM, supported by a marked reduction of in vivo switched memory B cells.

The lymphocyte immunophenotyping was assessed by flow cytometry. For flow cytometry analysis, the samples were incubated at 4°C for 20 minutes with the appropriate amount of monoclonal antibodies, following the manufacturer's instructions. The mixtures were lysed with ammonium chloride (NH_4Cl) lysing solution, then incubated at room temperature for 10 minutes, and finally washed with phosphate buffered saline. Samples were then acquired on a FACSCanto II flow cytometer and analysed with FACSDiva software (BD Bioscience). Fluorescein isothiocyanate-, phycoerythrin-, and peridin chlorophyll protein-coupled antibodies to the following cell-surface proteins were used for flow cytometry: CD19, CD45RA, CD27 (BD Pharmingen or Beckman Coulter) [11]. Results revealed an increase in CD19+ cells (44%), with complete lack of switched CD27+ B cells. Naïve T cells (CD45RA+) were normally found, but CD40-L expression on T lymphocytes after proper stimulation was low (0.2%), indicating a functional alteration of the machinery required for immunoglobulin class switch recombination (CSR). To rule out a non-random X-inactivation, responsible for a CD40-L defect in a female, a methylation assay was performed as previously described [12], and was found normal. Sequence analysis of NEMO and TACI (transmembrane activator and calcium-modulating cyclophilin ligand interactor) revealed no alterations. The mutational analysis of NEMO and TACI was carried out using the polymerase chain reaction test as described by Bardaro *et al.* [13,14]. Amplification was performed using specific primers for NEMO (forward-GAGGACCAATACCGAGCATC and JF3R reverse primers) and TACI (forward-GTGGTCACTTATTCTAAAGG and reverse-GCAGGATCTTGCCTGCGTC primers). Amplified products were automatically sequenced. ABI Prism dye terminator cycle sequencing kit and an ABI 377 Automated DNA Sequencer were used.

Treatment including daily physiotherapy with nebulized saline was started. Prolonged oral and/or intravenous antibiotics decided on the basis of sputum culture results were prescribed when required. During the follow-up, predominantly *Streptococcus pneumoniae* and *Haemophilus influenzae* were isolated at sputum cultures performed on a three-monthly basis. *Mycoplasma pneumoniae* infection was diagnosed at the age of 11 years. Overall, after the first pneumonia occurred during measles, she had 7 more episodes of chest X-ray

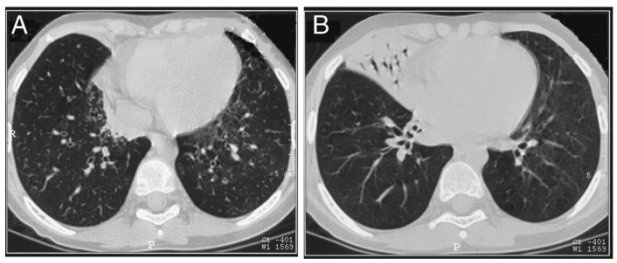

Figure 1 Chest HRCT: bronchiectasis in the left and right lower lobes (A) and a consolidation area in the middle lobe (B) may be observed.

documented pneumonia. At the age of 12 years she developed a skin lesion on the neck and the histological examination led to a diagnosis of cutaneous B-cells lymphoma successfully treated with at the Oncologic Department. Over the follow-up, the patient was visited about every 3 months, and serum IgM concentration, evaluated every 3-to-6 months, remained persistently elevated, while the low IgG levels progressively decreased over time eventually requiring, at the age of 12 years, the start of intravenous immunoglobulin replacement therapy because of serum IgG levels of 2.93 g/l (normal range, 6.40-19.09) (Figure 2). At present, the patient is 16-year old. Her clinical conditions are stable and daily physiotherapy with nebulised saline is still ongoing. Intravenous immunoglobulin replacement therapy is performed approximately every 3 months.

Discussion

Hyper IgM syndrome is a heterogeneous group of immune defects characterized by normal or increased production of IgM contrasting with a marked decrease or an absence of other isotypes (i.e., IgG, IgA, and IgE). The humoral immunodeficiency results in susceptibility to bacterial infections particularly affecting the respiratory tract. Patients with HIGM often present infections by opportunistic intracellular pathogens, such as *Pneumocystis carinii* [10,15], *Cryptosporidium* species [16], *Toxoplasma gondii* [17], and *Mycobacteria* species [18]. A common complication of both clinical and subclinical infections is represented by cholangiopathy, which may lead to the development of liver function tests alterations, sclerosing cholangitis, and cirrhosis that may eventually result in cholangiocarcinoma [16]. Chronic intestinal cryptosporidiosis may lead to weight loss, persistent diarrhea, and failure to thrive. Significant neurologic complications, such as cerebral toxoplasmosis [19,20] and cryptococcosis [21], are seen in 10-to-15% of affected males. Disseminated cytomegalovirus infection may be observed as presenting sign [22]. Moreover, neutropenia may often complicate CD40-ligand deficiency, while autoimmune complications are relatively common in patients with defects of CD40 signalling. Malignancies may also occur in patients with HIGM and usually affect the biliary tree [9,16] and the gastrointestinal tract in the form of neuroendocrine tumours [23]. As in other immunodeficiencies, patients also have an increased risk for lymphomas, particularly Hodgkin's disease associated with Epstein-Barr virus infection [24,25]. Indeed, AID deficiency causes the most frequent autosomal recessive alteration of CSR. Marked lymphoid hypertrophy represent a clinical feature of AID deficiency, even though malignant lymphoproliferation has not been ever described. Nevertheless, knock out mice for the UNG gene are prone to B cell lymphomas [26]. Furthermore, lymphomas are common in forms of HIGM due to DNA repair defects such as Ataxia-telangiectasia and Nijmegen Breakage syndrome. Allogeneic hematopoietic cell transplantation can be curative and feasible for the X-linked forms of HIGM without severe cryptosporidial infection and its related complications. If available, transplantation from either an HLA-matched sibling or an HLA-matched unrelated donor can be performed safely [27].

Mutations in five different genes involved in CSR have so far been associated with HIGM. X-linked forms are due to alterations of CD40-ligand and NEMO genes, while autosomal recessive forms are associated with mutations in CD40, AID, and UNG genes. The first recognized and most frequent form of HIGM, accounting for at least 70% of patients with CSR, is CD40-ligand deficiency [28,29]. Up to now, alterations of NEMO have

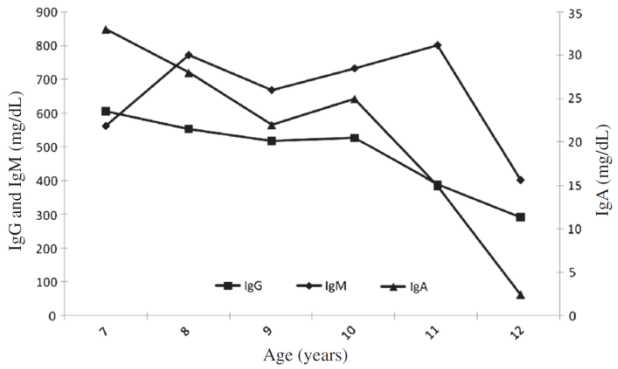

Figure 2 Immunoglobulin levels through the long-term follow-up: serum IgG and IgA progressively decreased to 2.93 g/l and 0.02 g/l, respectively, thus requiring intravenous immunoglobulin replacement therapy.

been excluded in our patient, while mutations in AID and UNG genes have not. However, we are planning to search for these genetic alterations in the near future.

Specialist referral to diagnose CSLD/bronchiectasis is recommended for children who have either two or more episodes of chronic wet cough per year, or chest radiographic abnormalities persisting for at least 6 weeks after appropriate therapy [30]. In previous large series, the majority of cases of CSLD appeared associated to extrinsic factors, especially childhood respiratory infections (severe pneumonia, pertussis, complicated measles and tuberculosis) that caused chronic endobronchial suppuration with or without bronchiectasis. Singleton et al. reviewed the case histories of 46 Alaskan native children with bronchiectasis born in the 1970s, and concluded that recurrent pneumonia was the major preceding medical condition leading to bronchial damage [31]. In a study by Eastham et al., previous pneumonia was the most common cause found in 93 cases of non-CF bronchiectasis [32]. Likewise, nearly 50% of children were found to have developed bronchiectasis after tuberculosis or severe pneumonia in a review study by Karakoc et al. [33]. Nowadays, with early immunization and the widespread use of antibiotics in childhood, post-infectious damage is likely to be less relevant than in non-vaccinated children [34]. Nonetheless, detailed investigations must be carried out to determine the underlying cause of the condition [35]. In CSLD, type, extent and severity of lung changes is evaluated by chest imaging techniques, including HRCT or magnetic resonance imaging [2,36].

Our case report highlights the importance to search for noninfectious extrinsic insults or intrinsic defects that predispose to bronchial inflammation or infection resulting in CSLD. These must include aspiration of irritants and congenital disorders, as immunodeficiencies and ciliary defects [37-39]. The identification of the underlying disorder is mandatory in that a delayed diagnosis is associated with more severe disease [40].

Consent

Written informed consent was obtained from the patient for publication of this Case report and any accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

Abbreviations

HIGM: Hyper-immunoglobulin M syndromes; CSR: Class switch recombination; CSLD: Chronic suppurative lung disease; HRCT: High resolution computed tomography; CF: Cystic fibrosis; PCD: Primary ciliary dyskinesia; NEMO: Nuclear factor (NF)-kB essential modulator; FEV_1: Forced expiratory volume in 1 second; FVC: Forced vital capacity.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

SM has made substantial contributions to conception and design, has been involved in drafting the manuscript, and has given final approval of the version to be published. MM has made substantial contributions to conception and design, has been involved in drafting the manuscript, and has given final approval of the version to be published. GG has made substantial contributions to acquisition of data, has been involved in drafting the manuscript, and has given final approval of the version to be published. ADG has made substantial contributions to acquisition of data, has been involved in revising the manuscript critically for important intellectual content, and has given final approval of the version to be published. LP has made substantial contributions to conception and design, has been involved in revising the manuscript critically for important intellectual content, and has given final approval of the version to be published. VM has made substantial contributions to acquisition of data, has been involved in revising the manuscript critically for important intellectual content, and have given final approval of the version to be published. MVU has made substantial contributions to acquisition of data, has been involved in revising the manuscript critically for important intellectual content, and have given final approval of the version to be published. MS has made substantial contributions to analysis and interpretation of data, has been involved in revising the manuscript critically for important intellectual content, and has given final approval of the version to be published. CP has made substantial contributions to conception and design and analysis and interpretation of data, has been involved in revising the manuscript critically for important intellectual content, and has given final approval of the version to be published. FS has made substantial contributions to conception and design and analysis and interpretation of data, has been involved in drafting the manuscript and revising it critically for important intellectual content, and has given final approval of the version to be published. All authors read and approved the final manuscript.

Author details

[1]Department of Pediatrics, Federico II University, via Pansini 5, Naples 80131, Italy. [2]Institute of Genetics and Biophysics Adriano Buzzati-Traverso, IGB-CNR, Naples 80131, Italy. [3]Department of Pediatrics, University Hospital of Parma, Parma, Italy.

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§3.3 Conclusive remarks

Through the study of these immunodeficiencies, enormous advances have been made in understanding the complex role of genes controlling immunity and other process in human, such as cancer and neurodegenetration.

It's noteworthy that immune and endocrine systems participate to an integrated network of soluble mediators that communicate and coordinate responsive cells to achieve effector functions in an appropriate fashion [205]. It has also recently been shown a novel dependence of GH signaling on the γc cytokines receptor in certain cell types, suggesting the interplay between endocrine and immune system [206]. There is evidence documenting that immune cells express GH-R [206]. GH-R can promote cell cycle progression of lymphoid cells and of a wide variety of other cells. Indeed, we documented a direct involvement of γc in self-sufficient growth and GH induced proliferation in a concentration dependent manner of the molecule [148].

Genes implicated in DNA repair may be implicated in both immune system and cancer predisposition as demonstrated by A-T phenotype. ATM plays a key role in the control of various cellular processes such as DNA repair, cell cycle progression, gene transcription, protein synthesis and degradation and apoptosis. This is may be considered a prototype of DNA repair defect syndromes.

Our data could provide the basis to develop in the near future new therapeutic strategies targeting this molecule in cancer therapy. Moreover, this information may also help understand undesired side effects of gene therapy trials.

These advances offer new insight into the mechanisms involved in immune surveillance against phatogens and tumoral cells, which might lead to develop new treatments, improving outcomes for affected subjects.

TECHNOLOGIES

§ Cells and cell cultures

Peripheral Blood Mononuclear cells (PBMC) were obtained from patients and healthy donors by Ficoll-Hypaque (Biochrom) density gradient centrifugation.

Keratinocytes were isolated by incubation of skin fragments in HBSS ($Ca2^+$ $Mg2^+$ free) containing 0.75% sodium bicarbonate, 100 mM HEPES (Gibco) supplemented with 25 μg/ml Gentamicin with Dispase II (20 mg/ml, Roche). Subsequent removal of epidermal sheet with tweezers, followed by incubation for 10 min at 37°C in 0.05% Trypsin and 0.5 mM EDTA (Gibco). Trypsin-EDTA were stopped adding the same volume of Fetal Bovine Serum (FBS). Cells were filtered through double layer gauze sterilized and then washed with DMEM containing 10% FBS. 5 x 10^6 keratinocytes were plated in 100 mm dish pre-treated with collagen coating solution containing HBSS with 100 μg/ml BSA, 20 mM HEPES pH 6.5, 30 ug/ml bovine type I collagen isolated from dermis (Nutacon). Cells were grown in supplemented Keratinocyte-SFM medium with 30 μg bovine pituitary extract and 0.2 ng/ml human rEGF (Gibco) and 100 IU/ml Penicillin and 100 μg/ml Streptomycin. The cultures were incubated at 37°C in the atmosphere supplemented with 5% CO_2, with the cell culture media changed daily. Fibroblasts were isolated by mincing of dermal skin fragments and were grown in Dulbecco's modified Eagle's medium (Invitrogen) and 10% FBS (Gibco), supplemented with 100 U/ml Penicillin and 100 μg/ml Streptomycin (Invitrogen). The cultures were incubated at 37°C in the atmosphere supplemented with 5% CO_2, with the cell culture media changed daily. Moreover, keratinocytes and fibroblasts were passaged fewer than 5 times before use in experiments.

$CD34^+$ hematopoietic stem cells were isolated by incubating 1-2 x 10^8 PBMCs in 300 μl of PBE with 100 μl of CD34 (QBEND 10)-conjugated magnetic beads (Multisort beads, Miltenyi Biotec), followed by incubation for 30 min at 4-8°C. After incubation, the cells were washed with ice-cold PBE (PBS/0.5% bovine serum albumin/5 mM EDTA) and processed through a column placed in a magnetic field and the target cells retained. After washing the column thoroughly

with ice-cold PBE, the target cells were recovered by removing the magnetic field and flushing the column with 1 ml of PBE. $CD34^+$ cells were then labeled with a CD34-FITC conjugated antibody for 15-20 min at room temperature.

B lymphoblastoid cell lines (BCLs) were generated by EBV immortalization of patients and healthy donors PBMC using standard procedures.

The human T-acute lymphoblastic leukemia cell line (Molt-4), the chronic myelogenous leukemia cell line (K-562), Burkitt lymphoma cell line and its isogenic derivatives (Raji and Rj225) were grown in RPMI-1640 (Lonza) supplemented with 10% fetal bovine serum (FBS; Gibco), 2 mmol/L L-glutammine (Gibco), and 50 μg/ml gentamycine (Gibco), and cultured at 37°C, 5% CO2. Serum starvation was used to synchronize tumor cells in the G0/G1-phase of the cell cycle. The cells were incubated in medium without FBS for 24 hours. In self-sufficient growth experiments, cells were cultured in DMEM/F12 (Lonza) without FBS and supplemented with 2 mmol/L L-glutammine.

Primary leukemic cell lines, consisting of acute lymphoblastic leukemia (ALL) cells, were obtained from aspirated bone marrow of 3 patients. Normal bone marrow cells were obtained from healthy donors and used as control cells.

§ SCID-like patient's profile

The patient was born at 42 weeks of gestation to unrelated healthy parents. At 8 months of age, the patient showed an autoimmune haemolytic anemia and a progressive decline of $CD4^+$ cells, resulting in a typical lymphocytopenic form of severe combined immunodeficiency. At the time of the study, lymphocytes were 3 x 10^9/l, but a severe lymphocyte functional impairment in the absence of HIV or any other viral infection was first noted as above described. IL-2Rγ alterations were ruled out. Thereafter, 2 bronchopneumonia and an interstitial pneumopathy occurred despite intravenous Ig replacement therapy and anti-infectious agents. Autoreactive antibodies toward smooth muscle, red and white cells were detected. During the follow-up, the patient developed a severely progressive active autoimmune hepatitis, diagnosed according the AIH international score, treated with Azathioprine (AZA) (1.5 mg/kg/day) and steroids. At 4 years of age the

patient died of disseminated interstitial pneumopathy, while the search for a HLA-matched donor was still pending.

§ Subjects with elevated IgE levels

Twenty patients with elevated IgE levels and history of allergy were enrolled into the study. Sixteen patients were affected by asthma, 3 of them also by rhinitis, and 4 had a history of atopic dermatitis. The patients divided in two subgroups on the basis of IgE levels: group A consisted of 10 patients, 10 males, range of age 5-15 years, with very high serum IgE levels (>2000kU/l, range 2152-5000 kU/l); group B consisted of 10 patients, 9 males, range of age 6-15 years, with high serum IgE levels (IgE value between the age specific mean±2SD and 2000 kU/l, range 93-1152 kU/l). Twenty healthy controls, 16 males range of age 6-15 years (IgE range 85-100 kU/l), were also studied. Informed consent was obtained when required. All patients enrolled into the study didn't receive any treatment, including steroid or non-steroid drugs, in the month before entering into the study. No difference was found between group A and B in either the number per year or the severity of allergic manifestations. In all patients, the clinical features persisted for more than 2 years.

The Hyper-IgE syndrome (HIES) was excluded by the absence of typical clinical and immunological features according to the clinical score for HIES. In particular, no recurrent skin infections, facial, skeletal and dentition anomalies were observed. Other conditions accompanied by elevated serum IgE concentration, including AIDS, helminths and parasitic infections were also excluded by clinical and laboratoristic features.

The study has been approved by the Institutional Review Board.

§ Generation of Th1-cell lines

Th1 cell lines were generated by stimulating PBMC with PHA (8 μg/ml) or, in a few experiments, with PHA + IFN-γ (1000 U/ml, ICN, Biomedical, OH) for 72 hours in complete tissue culture medium. These cells usually widely express high affinity IL-12R.

§ Membrane expression of β1 and β2 chains of IL-12R on T cells

After washing in PBS, cells were incubated for 20 minutes sequentially with murine anti-β1 or anti-β2 chain (25 μl) of IL-12R (kindly provided by Dr. Jerome Ritz, Dana Farber Cancer Institute, Boston, MA), IgG1 isotype control Ab, 10 μl FITC-conjugated goat anti-mouse IgG Ab (Becton Dickinson, San Jose, CA), and 5 μl anti-CD4 PE Ab (Becton Dickinson, San Jose, CA). After staining, the expression of IL-12Rβ1 and β2 on $CD4^+$ cells was determined with flow cytometer (Becton Dickinson) by gating on the $CD4^+$ population.

§ Preparation and characterization of porous scaffolds

3D porous scaffolds were developed by adapting the phase inversion and salt leaching technique. Scaffold morphology was preliminary investigated via FESEM Microscopy (Quanta FEG200, FEI). Specimens were fractured using a razor blade along preferential directions, parallel and perpendicular to the surface. Transverse and longitudinal sections were covered by a thin chromium layer (ca. 20 nm) by automatic sputtering (Emitech K575X) to afford a more efficient electron conductivity of the scaffold surface. The porosity was assessed in terms of pore size, shape and spatial distribution by images at different magnifications and fixed working distance (WD=10 mm). To obtain a quantitative estimation of the scaffold porosity, three different methods were used: weight measurements by gravimetric method (GM), 2D image analysis (2D-IA) and mercury intrusion porosimetry (MIP). The porosity was obtained by theoretical conditions. The 2D-IA evaluation of porosity features (porosity degree, pore size and spatial distribution) was performed by dedicated software (ImageJ 1.38b; NIH Freeware; National Institutes of Health, Bethesda). The porosity degree was evaluated from the total surface area of counted pores whereas the pore sizes were derived. Means and standard deviations of pore fraction and size were determined on 10 different SEM images. Porosity measurements by MIP were assessed to estimate the really interconnected pores and their specific pore surface. A mercury surface tension of 480 mN/m and a contact angle of 141.38° were used, while a pressure gradient

from 400 Pa up to 200 KPa was intruded to exactly count either micro and macropores according to the Washburn equation.

§ Scanning Electron Microscopy (SEM)

As for the investigation of biohybrid scaffold, cells were fixed for 2 h in 2,5% glutaraldehyde solution and dehydrated with sequential washes in 50%, 70%, 80%, 90% and 100% ethanol. The samples were air-dried overnight before the chromium sputtering. In this case, the accelerating voltage of the FESEM equipment (Quanta FEG200, FEI) was set at 5 kV, reducing the vacuum level into the chamber (LV or Low Vacuum mode), so preventing any negative interaction of electron beam with the cellular bodies.

§ siRNA transfection

The validated chemically modified oligonucleotides used as siRNA for IL2RG or random non-silencing nucleotides with no known specificity siRNA, used as negative control, were obtained from Invitrogen. These siRNAs were transfected at a concentration of 200 pmol/1x10^6 cells in a six well plate for 96 hours. The transfection was performed by lipid vector Lipofectamine 2000 kit (Invitrogen), according to the manufacturer's instructions. Preliminary experiments were performed to establish the silencing efficiency by testing two different oligonucleotides obtained from Invitrogen. The amount of protein expression reduction was calculated as follows: 1- (OD_{siRNA} x 100 / $OD_{control\ siRNA}$).

In self-sufficient growth experiments, BCLs were cultured in Dulbecco modified Eagle medium (DMEM)/F12 without FBS and supplemented with 2 mM/L L-glutamine.

§ Proliferative assay

Cell proliferation was analyzed by the thymidine incorporation assay.

For the evaluation in vitro of proliferative response to mitogens of PMBC, cells were stimulated with phytohaemagglutinin (PHA; 8 μg/ml), concanavalin A (ConA; 8 μg/ml), pokeweed (PWM, 10 μg/ml) (Difco Laboratories), phorbol-12-

myristate-13-acetate (PMA; 20 ng/ml) and ionomycin (0.5 mM) (Sigma Chemical Co). CD3 cross-linking (CD3 X-L) was performed by precoating tissue culture plates with 1 and 0.1 ng/ml purified anti-CD3 monoclonal antibody (Ortho Diagnostic).

To evaluate allogeneic response in patients with elevated IgE levels, cells (1 x 10^5) were stimulated with an equal amount of irradiated stimulator cells from controls in a standard one-way mixed lymphocyte reaction assay.

Cells were plated in triplicate at $1x10^5$ viable cells/well in 96-well plates (BD Biosciences), in 200 μl of complete medium for 4 days. Cultures were pulsed with 0.5 μCi ^{3}H-thymidine for 8 hours before harvesting and the incorporated radioactivity measured by scintillation counting.

In a few experiments complement components were inactivated through heating of serum samples at 56° C for 30 min before use. The percentage of inhibitory activity in the sera was calculated from the formula: (cpm of PHA-stimulated cultures containing 5% tested serum/cpm of PHA-stimulated cultures containing 5% FCS) x 100. Patients and normal IgG were purified using a protein G column according to the vendor's instructions (Pharmacia Biotech).

Cell proliferation was also analyzed by the CFSE dilution assay. Cells ($1x10^6$) were resuspended in 1 ml PBS-10% FBS and labeled with 1.7 μM CFSE (Molecular Probes). After 2 min in the dark at room temperature, cells were washed in FBS and PBS. After 6 hours cells were analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences).

Cell viability was determined using trypan blue staining. Cell survival was evaluated following stimulation with anti-Fas mAb (400 ng/ml; Upstate) for 6 hours.

§ Reagents, western blot and immuneprecipitation

Recombinant human GH (rGH) was obtained from Serono (Saizer 4). The enhanced chemiluminescence (ECL) kit was purchased from Amersham Biosciences. The Abs anti-γc, anti-JAK3, anti-beta-actin, anti-Bcl-2, anti-Bcl-XL, anti-histone 3 (H3), anti-phosphotyrosine, anti-STAT5, anti-STAT4 were

purchased from Santa Cruz Biotechnology. The Ab anti-caspase 3 was purchased from Cell Signaling Technology. Acrylamide and bisacrylamide were obtained from Invitrogen. Prestained molecular mass standards were obtained from Bio-Rad. Except where noted, other reagents were from Sigma-Aldrich.

Stimulated or unstimulated cells were washed with ice-cold phosphate buffer saline (PBS; Cambrex, Charles City, IA) and lysed in 100 μl of lysis solution containing 20 mM Tris (pH 8), 137 mM NaCl, 1% Nonidet P-40, 10 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 1 mM sodium orthovanadatum (Na3VO4), 5 μg/ml leupeptin and 5 μg/ml aprotinin on ice for 45 min. The cell lysates were stored at -80°C until processing. Proteins were separated on 12% SDS-PAGE. The membrane was then washed three times in wash buffer and incubated 1 h at room temperature or overnight at 4°C with the specific Ab. The membrane was then washed three times and an appropriate IgG HRPconjugated secondary Ab was used for the second incubation. After further washings, the membrane was developed with ECL-developing reagents, and exposed to x-ray films according to the manufacturer's instructions (Amersham Biosciences).

For immunoprecipitation, lysates were normalized for either protein content or cell number and precleared with protein G agarose beads (Amersham Biosciences). The supernatant was incubated with 2 microg/ml anti-JAK3 or polyclonal serum, followed by protein G agarose beads. The immunoprecipitates were separated on density gradient gels, followed by Western blotting. Proteins were detected using antibody for phosphotyrosine.

Densitometric analysis was performed after background equalization through the ImageJ software.

§ Confocal microscopy

After appropriate stimulation, as indicated, cells were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.0) for 30 min at room temperature and centrifuged in a Shandon Cytospin III (Histotronix) onto a glass slide and permeabilized by incubation in a 0.2% Triton X-100 solution for 20 min. BCLs were incubated for 1 h at room temperature with rabbit anti-STAT5 Ab in

PBS containing 1% BSA. After four washings for 5 min in PBS, the cells were incubated for 1 h at room temperature with FITC-conjugated donkey anti-rabbit IgG (Pierce) in PBS. After washing in PBS, the glass slides were mounted under a coverslip in a 5% glycerol PBS solution. The slides were analyzed by laser scanning confocal microscopy using a Zeiss LSM 510 version 2.8 SP1 Confocal System (Zeiss). At least 100 cells per condition were analyzed in each experiment to determine the rate of STAT5 nuclear translocation.

§ PCR and quantitative real-time PCR analysis

The cells cultured on each PCL construct were extracted from the scaffold by aspiration of the medium and flushing of the matrix at the different time point. RNA extraction was performed using RealTime ready Cell Lysis Kit (Roche Applied Science) according to the manufacturer's instructions. Total RNA was reverse transcribed using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science) according to the manufacturer's protocol using random hexameric primers. Amplification of the cDNAs was performed using the SYBR Green and analyzed with the Light Cycler480 (Roche) under the following conditions: 5 min of denaturation at 94°C followed by 55 cycles for 6 s of annealing at 62°C and 5 min of extension at 72°C. Real-time PCR utilized specific primers to amplify Ikaros, TAL1, Spi-B, PTCRA and RAG2 (Table 1). β-actin was used as a reference gene.

To evaluate the effect of γc on cell survival and proliferation, RNA was reverse-transcribed in the presence of SuperScript II RT (Invitrogen) and oligo(dT) primers (Invitrogen) at 50°C for 50 min and then at 85°C for 5 min to inactivate the enzymes. Amplification of the cDNAs was performed using the SYBR Green and analyzed with the Light Cycler480 (Roche). Primers are listed in Table 1. The PCR conditions comprised an initial denaturation at 94°C for 5 min, followed by 35 cycles at 62°C for 20 s and 72°C for 5 min. A dissociation procedure was performed to generate a melting curve for confirmation of amplification specificity. The results were normalized to beta-actin. The relative levels of gene expression are represented as $-\Delta Ct=(Ct_{gene}-Ct_{reference})$ and the fold

change in gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method (where Ct is cycle threshold), as previously described.

Gene	Primers sequence 5'-3'
Ikaros	5'- TCCCAAGTTTCAGGGAAGGA -3'
	5'- ACGACTCTGTCACTCTTGGAGCT -3'
TAL1	5'- TCTGAAGCAAGGCGGTGGAC -3'
	5'- GGAAGACCGTGCCGTCTTCA -3'
Spi-B	5'- TCGCCCTGGAGGCTGCAC -3'
	5'- CCCCCTCTGAATCAGGGTA -3'
PTCRA	5'- CATCCTGGGAGCCTTTGGT -3'
	5'- CCGGTGTCCCCCTGAGAG -3'
RAG2	5'- CCTGAAGCCAGATATGGTC -3'
	5'- GTGCAATTCACAGCTGGGCT -3'
Bcl-XL	5'-GTAAACTGGGGTCGCATTGT-3'
	5'-TGCTGCATTGTTCCCATAGA-3'
Cyclin D1	5'-AGGTCTGCGAGGAACAGAAGTG-3'
	5'-TGCAGGCGGCTCTTTTTC-3'
Cyclin D2	5'-CTGTGTGCCACCGACTTTAAGTT-3'
	5'-GATGGCTGCTCCCACACTTC-3'
Cyclin D3	5'-GCAGCGCCTTTCCCAACT-3'
	5'-TCAAAAGGAATGCTGGTGTATGTATC-3'
Cyclin A2	5'-CTGCTGCTATGCTGTTAGCC-3'
	5'-TGTTGGAGCAGCTAAGTCAAAA-3'
Cyclin B1	5'-CGGGAAGTCACTGGAAACAT-3'
	5'-AAACATGGCAGTGACACCAA-3‘
IL-2Rγ	5'-TGCTAAAACTGCAGAATCTGGT-3'
	5'-AGCTGGGATTCACTCAGGTTTG-3'
Beta-actin	5'-GACAGGATGCAGAAGGAGAT-3'
	5'-GACAGGATGCAGAAGGAGAT-3'

Table 1. Primers used for real-time qRT-PCR

To evaluate *IL-12Rβ2* chain expression, RNA was reverse transcribed into cDNA using Expand^{TM} Reverse transcriptase according to the manufacturer's protocol (Boehringer Manneheim). The cDNA was PCR amplified (94°C, 1 minute; 55°C, 1 minute; 72°C,1 minute for 30 cycles) using specific primers for IL-12Rβ2: sense primer GGAGAGATGAGGGACTGGT and antisense primer TCACCAGCAGCTGTCAGAG. To monitor the amount of RNA, β-actin mRNA expression was used. PCR products were separated in a 1% agarose gel and viewed after ethidium bromide staining.

§ Flow cytometry analysis

At defined time-points (0, 1, 2, 3 and 4 w), the cells cultured on each of the PCL construct were extracted from the scaffolds by aspiration of the medium and flushing of the matrices. Cells were exposed to directly-conjugated mouse anti-human monoclonal antibodies (mAbs) to asses hematopoietic stem cells with CD34-APC (BD Biosciences), CD45-APC-Cy7 (BD Biosciences), and lymphocyte precursors with CD7-PE (Immunological Sciences), CD1a-FITC (DAKO), CD3-PerCP (BD Biosciences), CD4-PE (BD Biosciences), CD8-PECy7 (Beckman Coulter). The cells were incubated with directly-labelled antibody clones at 4°C in the dark for 30 min, washed and resuspended in 100 ml PBS. The events in the displayed graphs and dot plots were gated by forward and side scatter to exclude dead cells. For analysis of early thymocyte subsets with CD7, CD3, CD4 and CD8 T-cell precursors were identified by gating on viable CD45+ cells. Analytical flow cytometry was performed using a BD FACS Canto II flow cytometer (BD Biosciences). Subsequent data processing and preparation for presentation was done using BD FACSDiva software.

§ Statistical analysis

All statistical analyses were performed using GraphPad Prism 4.00 and MedCalc for Windows. All data were expressed as mean ± standard deviation. Values of $p < 0.05$ were considered statistically significant.

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SUMMARY

The immune response is composed of a diverse network of defenses, including cellular components and soluble mediators. A proper immune response relies on the innate immunity, characterized by a rapid and nonspecific initial response to infections and later on the adaptive immunity, characterized by a specific response to a particular antigen. Failure of host defense may occur, causing the dysregulation of the immune system, in particular the onset of immunodeficiency, autoimmunity and cancer predisposition.

Primary immunodeficiencies comprise more than 200 different disorders that affect the development and the functions of the immune system. Many scientific papers have been published on the molecular and cellular basis of the immune response and on the mechanisms involved in the correct development of immune system components. Although today the genetic and molecular basis of the principal mechanisms involved in the immune response are well known, some aspect in this field remain unclear.

In this thesis, during the three years of my PhD program, I have contributed to elucidate "*Primary Immunodeficiencies: novel insight in pathogenesis and potential therapeutic approach*", through the combination of clinical, cellular, functional and molecular approaches.

In particular, my research work is focused on the deepening of the knowledge on thymic ontogeny, in particular on the study of the functional role of FOXN1 transcription factor in the development of the T-cell ontogeny and new strategy to develop an *in vitro* thymic organoid.

Moreover, I participate to give a contribution to better define the regulatory mechanisms of the immune system, with particular regard to the

central and peripheral tolerance, whom impairment function leads to autoimmunity.

Finally, I also participated to better define the role of the immune system genes, whom alteration induce the development of cancer predisposition, endocrine system failure and neurodegeneration. In particular I studied the role of γc in cell cycle progression, strongly related to its cellular amount and GH-R signaling, defining the basis of the physiological interaction between endocrine and immune systems, and the role ATM in the progressive neurological dysfunction.

Overall, all my studies were designed in order to clarify unsolved issues and unknown mechanisms underlying the functionality of the immune system. These results could be useful both in the clinical practice and in the basic research of immunedysregulation.

CURRICULUM VITAE
DR. LOREDANA PALAMAR0

Personal information

First name / Surname	**Loredana Palamaro**		
Address	**Via Molara, 29 - 80070 Barano of Ischia (NA) - Italy**		
Telephone(s)	+39-081-905142	Mobile:	+39-348-0139326
E-mail	lory983@libero.it		
Nationality	Italian		
Date of birth	January 02, 1983		
Gender	Female		

Education and training

Dates	From February 2010 – to 28 February 2013
Title of qualification awarded	PhD
Principal subjects/occupational skills covered	PhD student at the Doctoral Course in "Human Reproduction, Development and Growth" with a research project focused on the setting up of a new in vitro thymic organoid, through a 3D scaffold in the presence of skin derived cells, to support the differentiation of HSCs into T lineage-committed cells.
Name and type of organisation providing education and training	Unit of immunology, Department of Pediatrics at the "Federico II" University of Naples, via Pansini, 5 – 80131, Naples - Italy
Level in national or international classification	ISCED 7

Dates	September 2011
Principal subjects/occupational skills covered	Tutor for the students of the Biotechnology Science Faculty of the "Federico II" University of Naples consisting in: - Guidance and support for students enrolled in the first year; - Planning of a program of study and any questions of topics considered particularly difficult by the students.
Name and type of organisation providing education and training	Winner of the "Competition for the assignment of n. 530 checks for the tutor and teaching activities- Accademic Year 2010/2011- with the duty to commit 200 hours of activity for the students of the Biotechnology Science Faculty of the "Federico II" University of Naples, via Pansini, 5 – 80131, Naples – Italy

Dates	From 2007- to 2009
Title of qualification awarded	Bachelor in Medical Biotechnology (II level degree) at the "Federico II" University of Naples, with a thesis entitled: “Potenziale ruolo oncogenico della catena gamma in linee cellulari neoplastiche”. Vote: 110/110 and lode

Principal subjects/occupational skills covered	Internship at the Unit of Immunology, Department of Pediatrics of the "Federico II" University of Naples, Naples, Italy, focused on: - study of the common gamma chain and its potential oncogenic role in cell cycle progression of human malignant cell lines; the development of an in vitro thymic organoid.
Name and type of organisation providing education and training	"Federico II" University of Naples, via Pansini, 5 – 80131, Naples - Italy.
Level in national or international classification	ISCED 6

Dates	From 2006- to 2007
Title of qualification awarded	Bachelor in Biotechnology for Healthcare (I level degree) at the "Federico II" University of Naples with a thesis entitled: “Valutazione dell'interazione funzionale tra la catena gamma e il recettore del GH mediante saggio di neutralizzazione”. Vote: 104/110.
Principal subjects/occupational skills covered	Internship at the Unit of Immunology, Department of Pediatrics of the "Federico II" University of Naples, Naples, Italy, focused on the study of functional relationship between common gamma chain and growth hormone receptor and molecular characterization of apoptotic genes in patients with cluster of autoimmune disease (CAD).
Name and type of organisation providing education and training	"Federico II" University of Naples, via Pansini, 5 – 80131, Naples - Italy.
Level in national or international classification	ISCED 6

Personal skills and competences

Mother tongue(s)	ITALIAN
Other language(s)	ENGLISH

Self-assessment	Understanding		Speaking	
European level (*)	Listening	Reading	Spoken interaction	Spoken production
Language	B2	C1	B2	B2

(*) Common European Framework of Reference for Languages

Social skills and competences	Predisposition to work in a team of 5-6 persons by collaborating each other in a friendly manner and capacity to interact with other colleagues also in multicultural environments developed during my PhD studies.

Organisational skills and competences

- Capacity to design a scientific project including the economic budget. (See the attached list of application to grant proposals)
- Capacity to administrate small budgets for the daily work in a small lab.
- Capacity to coordinate students in their practice in laboratory also by follow them in the preparation of the thesis.
- Capacity to contribute to the coordination and management of the scientific revision of articles required by international scientific journals, as Cellular Immunology and Journal of Clinical Immunology.

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Technical skills and competences

- Knowledge of entrezgene, genecards, embl nucleotide sequence database and of the UCSC genome browser.
- Ability to perform a proliferative assay through the evaluation of thymidine incorporation by lymphocytes pre_stimulated with mitogens for diagnosis of the immunodeficiencies.

These competences were acquired during my training at the Department of Peditriatrics, where I also participated to clinical practice by diagnosing some immune disorders.

- Use of endnote 7.0 to format and add references to a manuscript.
- Use of Word, graphical softwares such as PowerPoint, Publisher,Photoshop, statitistical softwares such as Excel e GraphPad Prism and softwares to elaborate images such as ImageJ 1.42.

These competences were acquired during the training and the PhD at the Department of Peditriatrics. I also participated to the preparation of an entire scientific paper also by creating imagines and graphics and to the preparation of lessons, seminars and posters for congress and meetings (see the attached list of scientific production).

- Use of databases of scientific articles, in particular SCOPUS and ISI Web of Science and ability to perform the h-index and contemporary index calculi for the evaluation of scientific production.
- Use of the university program "Ugov-research catalogue" for the management of scientific publications of the university professors.

These competences were acquired during the PhD in the Department of Pediatrics, where I also contributed to the “Evaluation of the Quality of the Research” 2004-2010 (VQR 2004-2010) of the Department of Pediatrics of the "Federico II" University of Naples. Dr. Palamaro attended the advanced courses in the selection of the scientific production according to the criteria established by ANVUR in order to contribute significantly to better evaluation of the Department of Pediatrics - "Federico II" University.

Other skills and competences

SCIENTIFIC SKILLS

- Cell cultures
- Primary and continue human cell lines manipulation.
- Primary culture of skin derived fibroblast preparation.
- B lymphocyte immortalization with EBV infection.
- Scaffold preparation and characterization
- CD34+ isolation with microbeads (MACS)
- Proliferation assay (thymidine, CFSE, MTS)
- Death assay (Trypan blue, Annexin V, Pridium Iodure)
- Western blot
- Immunofluorescence
- Flow cytometry
- DNA and RNA extractions (cells and tissues)
- PCR and Sequencing analysis
- Reverse transcriptase and Real_time PCR
- Transfection and RNA interference with lipofectamine

Additional information

SCIENTIFIC INTERESTS

Major fields of my scientific interests are as follows:

- Regulatory mechanisms governing lymphocyte cell proliferation, activation and cell death. In particular, the study role of γc in cell cycle progression, strongly related to its cellular amount;
- Novel aspects in immunodeficiencies, with a particular regard to Severe Combined immunodeficiency (SCID) and molecular analysis of genes whose mutations are responsible for certain immunodeficiencies;
- T-cell ontogeny process in human. In particular, the focus is to develop an in vitro thymic organoid and to study the functional role of FOXN1 transcription factor in the development of the T_cell ontogeny.
- Studies of previously unappreciated relationships between receptor signaling systems in the pathogenesis of SCIDs and signal transduction in physiology and human diseases affecting the immune system;
- Primary Immunodeficiencies: definition of novel therapeutical strategies for the treatment of Ataxia_Teleangiectasia.

SCIENTIFIC SOCIETIES:

Sirp, Società Italiana di Ricerca Pediatrica: Junior member.

Annexes

1. **APPLICATION TO GRANTS PROPOSALS**
2. **SCIENTIFIC PRODUCTION**

1. APPLICATIONS TO GRANT PROPOSALS

(Dr. Loredana Palamaro)

• Call for Application in Malattie Rare 2009 (Minister of Health) with a project entitled: "Study of the T-cell ontogeny defect in the murine and human Nude/SCID and in the DiGeorge syndromes".

• Telethon Grant Proposals-Call for Applications 2010, with a project entitled: "Characterization of the T−cell ontogeny defect in the human athymic models of Nude/SCID and DiGeorge syndromes".

• Call for Application in Ricerca Scientifica in ambito biomedico - Fondazione Cariplo 2010, with a project entitled: "Functional cellular and molecular studies to elucidate the immune pathogenesis of multiple autoimmune manifestations of childhood".

• Call for Application in Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale - Bando MIUR 2009, with a project entitled: "Studio dell'ontogenesi delle cellule T in modelli umani di atimia ed autoimmunità: Allestimento di "scaffold" tridimensionali per la generazione in vitro di cellule T e Treg da precursori ematopoietici".

• Call for AIRC Application 2010, with a project entitled: "Potential oncogenic role of the X-SCID gamma chain gene".

• SIRPED – Società Italiana di Ricerca Pediatrica 2011, with a project entitled: "Modulation of molecular mechanism implicated in the high predisposition to infection and cancer in children with Ataxia-Teleangiectasia".

• Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale - Call for MIUR Application 2010-11, with a project entitled: "Sviluppo di approcci innovativi diagnostici e terapeutici per le immunodeficienze primitive".

• Futuro in Ricerca – Call for MIUR Application 2012, with a project entitled: "Studio dei meccanismi che regolano l'omeostasi dei linfociti B e T nelle Immunodeficienze Primitive: difetti primitivi a carico dei linfociti T e l'Immunodeficienza Comune Variabile (CVID) come modelli".

• Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale - - Call for MIUR Application 2012, with a project entitled: "Sviluppo di approcci innovativi diagnostici e terapeutici per le immunodeficienze primitive"

• Futuro in Ricerca – - Call for MIUR Application 2013, with a project entitled: "Caratterizzazione del potenziale ruolo oncogenico della γc, attraverso lo studio della sua attività modulatoria su geni legati alla crescita, al ciclo cellulare, alla sopravvivenza ed attraverso lo studio della regolazione post-trascrizionale mediata da miRNA correlati alla γc".

• Telethon Grant Proposals-Call for Applications 2010, with a project entitled: "Evaluation of the cytoplasmic role of ATM kinase in the autophagy-lysosomal pathway and its pathogenic implication in Ataxia Telangiectasia: potential modulatory effect of Betamethasone".

2. SCIENTIFIC PRODUCTION
(Dr. Loredana Palamaro)

LIST OF PUBLICATIONS:

1. Amorosi S., Russo I., Amodio G., Garbi C., Vitello L., **Palamaro L.,** Adriani M, Vigliano I., Pignata C. The cellular amount of the common g chain influences spontaneous or induced cell proliferation. J Immunol 182: 3304-3309, 2009.
2. Vigliano I., Amorosi S., Fusco A., Vitiello L., **Palamaro L.,** Maio F:, Gallo V., Pignata C. Gamma chain expression level influences spontaneous cell proliferation in different malignant hematopoietic cell lines. Clin Immunol. 135: 326, 2010. (Abstract)
3. Fusco A., Vigliano I., **Palamaro L.,** Cirillo E., Aloj G., Piscopo G., Giardino G., Pignata C. Altered signaling through IL-12 receptor in children with very high serum IgE levels. Cell Immunol 265: 74–79, 2010.
4. Vigliano I., Fusco A., **Palamaro L.,** Aloj G., Cirillo E., Salerno M.C., Pignata C. Gamma chain transducing element: a shared pathway between endocrine and immune system. Cell Immunol 269: 10-15, 2011
5. **Palamaro L.,** Guarino V., Scalia G., Antonimi D., De Falco L., Vigliano I., Fusco A., Vitiello L., Giardino G., Caterina M., Del Vecchio L., Ambrosio L., Pignata C. 3-dimensional poly(3-caprolactone) scaffold containing skin-derived fibroblasts and keratinocytes supports in vitro HSCs differentiation in T-lineage-committed cells. J Clin Immunol 31 (Suppl 1):S1-S71, 2011 (Abstract)

6. Vigliano I., Fusco A., Panico L., Gorrese M., **Palamaro L.,** Bianchino G., Del Vecchio L., Pignata C. Potential extrathymic lymphopoiesis in a athymic human fetus carrying homozygous FOXN1 mutation. J Clin Immunol 31 (Suppl 1):S1-S71, 2011 (Abstract)

7. **Palamaro L.,** Vigliano I., Giardino G., Cirillo E., Aloj G., Romano R., Pignata C. SCID-like phenotype associated with an inhibitory autoreactive immunoglobulin. J Investig Allergol Clin Immunol 22:67-70, 2012

8. Vigliano I., **Palamaro L.,** Bianchino G., Fusco A., Vitiello L., Grieco V., Romano R., Salvatore M., Pignata C. Role of the common γ chain in cell cycle progression of human malignant cell lines. Int Immunol 24:159-157, 2012

9. Romano R., **Palamaro L.,** Fusco A., Iannace L., Maio S., Vigliano I., Giardino G., Pignata C. From murine to human nude/SCID: the thymus, T-cell development and the missing link. Clin Dev Immunol 2012:467101, 2012

10. Capalbo D., Melis D., De Martino L., **Palamaro L.,** Riccomagno S., Bona G., Cordeddu V., Pignata C., Salerno M. Noonan-like syndrome with loose anagen hair associated with growth hormone insensitivity and atypical neurological manifestations. Am J Med Genet A. 158A:856-60, 2012

11. Capalbo D., Giardino G., De Martino L., **Palamaro L.,** Romano R., Gallo V., Cirillo E., Salerno M., Pignata C. Genetic basis of altered central tolerance and autoimmune diseases: a lesson from AIRE mutations. Int Rev Immunol. 31:344-362, 2012

12. **Palamaro L.,** Giardino G., Santamaria F., Ramenghi U., Dianzani U., Pignata C. Altered regulatory mechanisms governing cell survival in children affected with clustering of autoimmune disorders. Ital J Pediatr. 38:42, 2012

13. **Palamaro L.,** Giardino G., Santamaria F., Romano R., Fusco A., Montella S., Salerno M., Ursini M.V., Pignata C. Interleukin 12 receptor deficiency in a child with recurrent bronchopneumonia and very high IgE levels. Ital J Pediatr. 38:46, 2012

14. Montella S., Maglione M., Giardino G., Di Giorgio A., **Palamaro L.,** Mirra V., Ursini M.V., Salerno M., Pignata C., Caffarelli C., Santamaria F. Hyper IgM syndrome presenting as chronic suppurative lung disease. Ital J Pediatr. 38:45, 2012

15. Romano R., **Palamaro L.,** Parenti G., Salerno M., Fusco A., Vajro P., Capalbo D., Ranieri B., Naddei R., Pignata C. Networking between γc and GH-R signaling in the control of cell growth. Curr Signal Transduct Ther. 8:67-73, 2012

16. Giardino G., Fusco A., Romano R., Gallo V., Maio F., Esposito T., **Palamaro L.,** Parenti G., Salerno M.C., Vajro P., Pignata C. Betamethasone therapy in Ataxia-Telangiectasia: unraveling the rationale of this serendipitous observation on the basis of the pathogenesis. Eur J Neurol. In press, 2012

17. **Palamaro L.,** Guarino V., Scalia G., Antonini D., De Falco L., Bianchino, G., Fusco A., Romano R., Grieco V., Missero C., Del Vecchio L. Ambrosio L., Pignata C. Molecular signature of the T-cell commitment in an in vitro three-dimensional organoid mimicking the thymic microenvironment. J Clin Immunol. 32 (Suppl 1):S310, 2012 (Abstract)

18. Romano R., Ferrentino R., Pane L.S., **Palamaro L.,** Fusco A., Marques J.G., Sousa A.B., de Sousa A.E., Ursini M.V., Baldini A., Pignata C. Preliminary steps of fibroblasts reprogramming to develop mTECs from control or FOXN1$^{-/-}$ fibroblasts. J Clin Immunol. 32 (Suppl 1):S332, 2012 (Abstract)

19. Improda N., Alessio M., Capalbo D., Russo G., D'Acunzo I., **Palamaro L.,** Pignata C., Salerno M. Salerno Acute adrenal failures as the presenting feature of primary antiphospholipid syndrome in a child. Ital J Pediatr. 38:49, 2012

20. Capalbo D., Scala MG., Melis D., Minopoli G., Improda N, **Palamaro L.,** Pignata C., Salerno M. Clinical Heterogeneity in two patients with Noonan-like Syndrome associated with the same SHOC2 mutation. Ital J Pediatr. 38:48, 2012

21. Gallo V., Giardino G., Capalbo D., **Palamaro L.,** Romano R., Santamaria F., Maio F., Salerno M., Vajro P., Pignata C. Alterations of the autoimmune regulator transcription factor and failure of central tolerance: APECED as a model. Expert Rev Clin Immunol. 9:43-51, 2013

22. van de Vosse E., van Dissel JT., Palamaro L., Giardino G., Santamaria F., Romano R., Fusco A., Montella S., Salerno M., Ursini MV., Pignata C. The R156H variation in IL-12Rbeta1 is not a mutation. Ital J Pediatr. 39:12, 2013

23. **Palamaro L**., Romano R., Fusco A., Giardino G., Gallo V., Pignata C. FOXN1 in cell development and human diseases. Int Rev Immunol. In press, 2013

MEETING ABSTRACTS AND COMMUNICATIONS:

1. Amorosi S., Russo I., Amodio G., Garbi C., Vitiello L., **Palamaro L.,** Adriani M., Vigliano I., Pignata C. The common γ chain provides spontaneous and GH-dependent cell cycle progression, related to its cellular amount. Day of Immunology. Primary Immunodeficiencies: emerging challenges. Napoli 29 Aprile 2008 (Oral presentation)

2. Amorosi S., Russo I., Amodio G., Garbi C., Vitiello L., **Palamaro L.,** Vigliano I., Pignata C. g-chain provides a spontaneous and GH dependent sgnal fo cell cycle progression related to its cellular amount. Annual Meeting for the Federation of Clinical Immunology Societies, Boston 5-9 June 2008 (Oral presentation)

3. Vigliano I., **Palamaro L.,** Vitiello L., Fusco A., Cirillo E., Aloj G., Gallo V., Pignata C. Correlazione diretta tra livelli di espressione di IL-2Rγ e proliferazione cellulare spontanea in linee cellulari maligne ematopoietiche. 12° Congresso Nazionale SIAIP. Bari, 14-17 Aprile 2010 (Poster)

4. Fusco A., Gorrese M., Aloj G., Vigliano I., **Palamaro L.,** Cirillo E., Giardino G., Del Vecchio L. and Pignata C. Caratterizzazione del difetto di ontogenesi T nei due modelli umani di atimia, la sindrome Nude/SCID e la sindrome di DiGeorge. 12° Congresso Nazionale SIAIP. Bari, 14-17 Aprile 2010 (Poster)

5. Vigliano I., **Palamaro L.,** Vitiello L., Amorosi S., Fusco A. and Pignata C. Gamma chain expression levels influence spontaneous cell proliferation in different malignant hematopoietic cell lines. In: Day of Immunology "Autoimmunity: from basic immunology to clinics". Napoli, 29 Aprile 2010 (Oral presentation)

6. Vigliano I., Amorosi S., Fusco A., Vitiello L., **Palamaro L.,** Maio F:, Gallo V., Pignata C. Gamma chain expression level influences spontaneous cell proliferation in different malignant hematopoietic cell lines. First CIS North American Primary Immune Deficiency National Conference. Philadelphia 20-23 Maggio, 2010. (Poster)

7. Fusco A., Panico L., Gorrese M., Vigliano I., **Palamaro L.,** Del Vecchio L. and Pignata C. Prenatal T-cell ontogeny in human Nude/SCID fetus and extrathymic lymphopoiesis. XIVth meeting of the European Society for Immunodeficiencies, Istanbul 6-10 Ottobre, 2010 (Poster)

8. Vigliano I., Bianchino G., **Palamaro L.,** Grieco V., Vitiello L., Fusco A., Cirillo E., Salvatore M. and Pignata C. Effects of gamma chain on cyclins expression and cell

cycle progression in malignant hematopoitic cell lines. XIVth meeting of the European Society for Immunodeficiencies, Istanbul 6-10 Ottobre, 2010 (Poster)

9. Cirillo E., Fusco A., Vigliano I., **Palamaro L.,** Aloj G., Giardino G. , Gallo V., Maio F. , Valentino L. , Cosentini E. and Pignata C. Severe combined immunodeficiency phenocopy associated with an inhibitory autoreactive immunoglobulin. XIVth meeting of the European Society for Immunodeficiencies, Istanbul, 6-10 Ottobre, 2010 (Poster)

10. **Palamaro L.,** Guarino V., Scalia G., Antonimi D., DeFalco L., Vigliano I., Fusco A., Vitiello L., Giardino G., Caterina M., Del Vecchio L., Ambrosio L., Pignata C. 3-dimensional poly(3-caprolactone) scaffold containing skin-derived fibroblasts and keratinocytes supports in vitro HSCs differentiation in T-lineage-committed cells. Advanced School in Primary ImmunoDeficiency Disease Chicago 18-19 Maggio 2011 (Selected oral presentation)

11. Vigliano I., Fusco A., Panico L., Garrese M., **Palamaro L.,** Bianchino G., Del Vecchio L., Pignata C. Potential extrathymic lymphopoiesis in a athymic human fetus carrying homozygous FOXN1 mutation. Advanced School in Primary ImmunoDeficiency Disease Chicago 18-19 Maggio 2011 (Selected oral presentation)

12. **Palamaro L.,** Guarino V., Scalia G., Antonini D., De Falco L., Bianchino, G., Fusco A., Romano R., Grieco V., Missero C., Del Vecchio L., Ambrosio L., Pignata C. Molecular signature of the T-cell commitment in an in vitro three-dimensional organoid mimicking the thymic microenvironment. XVth meeting of the European Society for Immunodeficiencies, Florence 03-06 Ottobre 2012 (poster)

13. Romano R., Ferrentino R., Pane L.S., **Palamaro L.,** Fusco A., Marques J.G., Sousa A.B., de Sousa A.E., Ursini M.V., Baldini A., Pignata C. Preliminary steps of fibroblasts reprogramming to develop mTECs from control or FOXN1-/- fibroblasts. XVth meeting of the European Society for Immunodeficiencies, Florence 03-06 Ottobre 2012 (Poster)

AWARDS:

1. Amorosi S., Russo I., Amodio G., Garbi C., Vitiello L., **Palamaro L.,** Vigliano I., Pignata C. γ-Chain provides a spontaneous and gh dependent sgnal fo cell cycle progression related to its cellular amount. Annual Meeting For The Federation Of Clinical Immunology Societies, Boston 5-9 June 2008

2. Vigliano I., Amorosi S., Fusco A., Vitiello L., **Palamaro L.,** Maio F:, Gallo V., Pignata C. Gamma chain expression level influences spontaneous cell proliferation in different malignant hematopoietic cell lines. First CIS North American Primary Immune Deficiency National Conference. Philadelphia 20-23 Maggio, 2010.

3. **Palamaro L.,** Guarino V., Scalia G., Antonini D., Giardino G., De Falco L., Vigliano I., Fusco A., Missero C., Del Vecchio L., Ambrosio L., Pignata C. 3-dimensional poly(3-caprolactone) scaffold containing skin-derived fibroblasts and keratinocytes supports in vitro HSCs differentiation in T lineage-committed cells. Advanced School in Primary Immune Deficiency. Chicago, 18-19 Maggio, 2011.

4. Vigliano I., Fusco A., Panico L., Gorrese M., **Palamaro L.,** Bianchino G., Del Vecchio L., Pignata C. Potential extrathymic lymphoiesis in an athymic human fetus carrying homozygous FOXN1 mutation. Advanced School in Primary Immune Deficiency. Chicago, 18-19 Maggio, 2011.